



Europäisches Patentamt

European Patent Office

Office européen des brevets

⑪ Publication number:

0 101 309

B1

⑫

## EUROPEAN PATENT SPECIFICATION

⑬ Date of publication of patent specification: 16.01.91

⑮ Int. Cl. 5: C 12 N 15/16, C 07 K 13/00,  
C 07 K 7/00

⑯ Application number: 83304662.6

⑰ Date of filing: 11.08.83

⑭ Divisional application 88104503 filed on 21.03.88.

⑯ Molecular cloning and characterization of a gene sequence coding for human relaxin.

⑬ Priority: 12.08.82 AU 5352/82

⑮ Proprietor: HOWARD FLOREY INSTITUTE OF  
EXPERIMENTAL PHYSIOLOGY AND MEDICINE  
c/o University of Melbourne  
Parkville Victoria (AU)

⑬ Date of publication of application:  
22.02.84 Bulletin 84/08

⑯ Inventor: Hudson, Peter John  
1 Sefton Street, Bulleen  
Victoria (AU)  
Inventor: Shine, John  
107 Barnett Close, Swinburne Hill  
Australian Capital Territory (AU)  
Inventor: Niall, Hugh David  
3 Bendigo Avenue, Elwood  
Victoria (AU)  
Inventor: Tregear, Geoffrey William  
62 Hawthorn Grove, Hawthorn  
Victoria (AU)

⑬ Publication of the grant of the patent:  
16.01.91 Bulletin 91/03

⑯ Representative: Brown, John David et al  
FORRESTER & BOEHMERT Widenmayerstrasse  
4/1  
D-8000 München 22 (DE)

⑯ Designated Contracting States:  
AT BE CH DE FR GB IT LI LU NL SE

⑯ References cited:  
EP-A-0 086 649 DE-A-3 102 487

Nature, vol.291, 1981, pp127-131;  
Genetic Engineering, Commercial Opportunities  
in Australia, Proceedings of a Symposium in  
Sydney, 18-20 Nov 1981, pp135-138, Australian  
Government Public Service

NATURE, vol. 301, no. 5901 February 17, 1983,  
Macmillan Journals Ltd. Basingstoke, GB, P  
Hudson et al.: "Structure of a genomic clone  
encoding biologically active human relaxin",  
pages 628-631

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Courier Press, Leamington Spa, England.

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② References cited:

**TELEGEN TECHNICAL FEATURE 82000533,**  
**Abstract 31808, 1982 Environment Information**  
**Center New York, US, H NIALL: "Hormones and**  
**genetic engineering" paper presented at the**  
**genetic engineering symposium in Sydney,**  
**November 18-20, 1981**

**DNA, vol. 1, no. 2, 1982, Mary Ann Liebert Inc.**  
**New York, US, J HALEY et al.: "Porcine Relaxin:**  
**Molecular Cloning and cDNA Structure", pages**  
**155-162**

**NATURE, vol. 267, 1977, pages 544-546,**  
**Macmillan Journals Ltd. Basingstoke, GB; R**  
**JAMES et al.: "Primary structure of porcine**  
**relaxin: homology with insulin and related**  
**growth factors"**

## Description

This invention relates to the molecular cloning and characterization of the gene sequence coding for human relaxin. The invention is also concerned with recombinant DNA techniques for the preparation of 5 human relaxin, prorelaxin and preprorelaxin.

More specifically, this invention relates to an isolated and purified ("cloned") human gene coding for prorelaxin, preprorelaxin, and the A and/or B and/or C peptide chains of human relaxin, methods for isolating and purifying the genes and methods for transferring the genes to and replicating the genes in a host cell. The cloned genes are expressed by the host cell when fused with a host-expressible prokaryotic 10 or eukaryotic gene. The genes are thus useful in the production of human relaxin for therapeutic purposes.

The invention also relates to the peptides human relaxin, prorelaxin and preprorelaxin, to the individual peptide chains which comprise these sequences and to modified forms of these peptides.

The invention further relates to modified genes coding for the individual relaxin chains and for the above-mentioned modified forms.

15 Note: References referred to by number used in the following description are collected at the end of the description.

Pioneering work by Hisaw (1) suggested an important role for the peptide hormone relaxin in mammals through its effects in dilating the cervix and softening the pubic symphysis, thus facilitating the birth process. Relaxin is synthesized and stored in the corpora lutea of ovaries during pregnancy and is released 20 into the blood stream prior to parturition. The availability of ovaries has enabled the isolation and amino acid sequence determination of relaxin from pig (2, 3) rat (4) and shark (5). The biologically active hormone consists of two peptide chains (known as the A and B chains) linked together by disulphide bonds, two inter-chain and one intra-chain. The structure thus closely resembles insulin in the disposition of disulphide bonds which has led to speculation of a common ancestral gene for these hormones (2, 3).

25 A process for the preparation of human relaxin from chorionic membrane has been disclosed in DE-A-3102487.

Recombinant DNA techniques have been applied to the isolation of cDNA clones for both rat and porcine relaxins (6), (7), see also European Patent Application No. 0086649. Synthetic undecamer nucleotides, prepared on the basis of amino acid sequence information, were used as primers for the 30 synthesis of cDNA probes greatly enriched in relaxin cDNA sequences which identified relaxin cDNA clones in libraries derived from ovarian tissue. The relaxin structural gene was found to code for a single chain precursor which resembles preproinsulin in the overall configuration, i.e., signal peptide/B chain/C peptide/A chain.

35 Pig and rat preprorelaxins contain an unexpectedly large connecting peptide of 105 and 104 residues respectively in comparison to rat insulin with a C peptide of about 30 residues. A high degree of sequence homology in the C-peptide of rat and pig relaxin suggests a role beyond simply ensuring the correct disulphide bond formation of the A and B chains. We predicted that structural constraints on sequence 40 divergence applying during evolution would have resulted in the C-peptide region having a similarly high degree of sequence homology in the human relaxin gene. Accordingly, as described hereinafter, we have used probes based on the C-peptide region of porcine rather than rat relaxin in the selection of the human relaxin gene because the accumulation of protein sequence data indicated that human proteins are in general less divergent from porcine than from rat proteins (8).

Although it has been the long term goal of several groups to determine the structure of human relaxin and so establish a route to clinical intervention in cases of difficult labour, the limited availability of human 45 ovaries during pregnancy has prevented direct amino acid sequence determination. Our approach was to screen directly for the human relaxin gene in a genomic library using a region of the porcine relaxin cDNA as a probe. This approach resulted in the successful identification of genomic clone from which the structure of the entire coding region of preprorelaxin has been determined.

50 It is now believed that either or both the presently-described gene which we have designated "H1" and the "H2" gene described in our copending European application No. EP-A-0112149 are expressed in human reproductive tissue, for example ovary and placenta, and/or other tissues including but not limited to gut, brain and skin, since both genes express peptides with relaxin-like activity.

The corpora lutea of the ovary as well as decidual and placental tissues are the most likely sites for 55 expression of relaxin-related genes. However, in view of the wide distribution of many peptide hormones it is highly likely that the relaxin gene is also expressed in non-reproductive tissues, including brain and the gastrointestinal tract. Relaxin has the general properties of a growth factor and is capable of altering the nature of connective tissue and influencing smooth muscle contraction. We believe that one or both of the gene structures described in this and the copending patent application EP-A-0112149 to be widely distributed in the body. We suggest that the relaxin peptides expressed from these genes will play an 60 important physiological role in addition to their well documented hormonal function during reproduction.

The following abbreviations are used in this description.

H1 — the relaxin gene described herein, being deduced from a genomic clone.

H2 — the relaxin gene described in copending Application No. EP-A-0112149 being deduced from a cDNA clone.

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5  
 DNA — deoxyribonucleic acid  
 RNA — ribonucleic acid  
 cDNA — complementary DNA  
 (enzymatically  
 synthesized  
 from an mRNA sequence)  
 mRNA — messenger RNA

A — Adenine  
 T — Thymine  
 G — Guanine  
 C — Cytosine  
 U — Uracil

10 The coding relationships between nucleotide sequence in DNA and amino acid sequence in protein are  
 collectively known as the genetic code, which is set out below.

First position (5' end)		Second position			Third position (3' end)
		U.	C	A	G
20	U	Phe	Ser	Tyr	Cys
		Phe	Ser	Tyr	Cys
		Leu	Ser	Stop	Stop
		Leu	Ser	Stop	Trp
25	C	Leu	Pro	His	Arg
		Leu	Pro	His	Arg
		Leu	Pro	Gln	Arg
		Leu	Pro	Gln	Arg
30	A	Ile	Thr	Asn	Ser
		Ile	Thr	Asn	Ser
		Ile	Thr	Lys	Arg
		Met	Thr	Lys	Arg
35	G	Val	Ala	Asp	Gly
		Val	Ala	Asp	Gly
		Val	Ala	Glu	Gly
		Val	Ala	Glu	Gly

45 The abbreviations used for the amino acids in the table are identified as follows.

Phenylalanine	(Phe)	Histidine	(His)
Leucine	(Leu)	Glutamine	(Gln)
Isoleucine	(Ile)	Asparagine	(Asn)
Methionine	(Met)	Lysine	(Lys)
Valine	(Val)	Aspartic acid	(Asp)
Serine	(Ser)	Glutamic acid	(Glu)
Proline	(Pro)	Cysteine	(Cys)
Threonine	(Thr)	Tryptophan	(Try)
Alanine	(Ala)	Arginine	(Arg)
Tyrosine	(Tyr)	Glycine	(Gly)

60  
 65 Each 3-letter codon represented in the table, e.g., AUG, CAU (otherwise known as a deoxynucleotide triplet or nucleotide triplet) corresponds to a trinucleotide of mRNA, having a 5'-end on the left and a 3'-end

on the right. The letters stand for the purine or pyrimidine bases forming the nucleotide sequence. All DNA sequences given herein are those of the strand whose sequence corresponds to the mRNA sequence, with thymine (T) substituted for uracil (U).

In the following discussion reference will be made to the accompanying drawings in which:

5 Figure 1 is an abbreviated restriction enzyme map of the two genomic clones mentioned below;

Figure 2 shows how to align figures 2a and 2b which when aligned give the mRNA sequence of the coding region of the human relaxin gene and the amino-acid sequence of human preprorelaxin; and

Figure 3 shows a comparison of the human preprorelaxin and mRNA sequences with the corresponding sequences for porcine preprorelaxin.

10 The original source of genetic material was a library of human genomic clones. Screening of this library using pig relaxin cDNA probes yielded two clones containing coding sequences of human relaxin.

The mRNA sequence shown in Figures 2 and 3, was determined by the methods described hereinafter. It will be seen that a single intron of 3.4kb interrupts the coding region of the connecting (C) peptide. The structure of human preprorelaxin was deduced from the genomic sequence by comparison with the 15 homologous structures of pig and rat relaxin. Confirmation of the A and B peptide chain structures has been provided by synthesis and chain recombination *in vitro* which produces a material which is biologically active in the uterine contraction assay.

The mode of *in vitro* processing of the preprorelaxin is not yet fully known but by analogy with pig relaxin cleavage of the signal peptide would be expected to occur at the Ala<sup>1</sup>-Lys<sup>1</sup> bond. Similarly excision 20 of the C peptide is predicted to occur at Leu<sup>32</sup>-Ser<sup>33</sup> and Arg<sup>136</sup>-Arg<sup>137</sup>, thus giving the B and A chains of respectively 32 and 24 residues.

As noted in our studies on pig relaxin, there are core sequences in the pig relaxin B and A chains which contain all the essential elements for biological activity. Our synthetic studies on the human relaxin chain show similar results, as set out in more detail hereinafter.

25 According to one aspect of the present invention, there is provided a gene for the expression of human preprorelaxin.

More specifically, this aspect of the invention provides a double-stranded DNA fragment for the expression of human preprorelaxin, which comprises a coding strand and a complementary strand corresponding to the complete mRNA (codons -25 to 160) sequence shown in Figure 2 of the 30 accompanying drawings.

The invention also includes any sub-unit of the preprorelaxin gene sequence described herein, or any equivalent of the said sequence or sub-unit. Among the sub-units to be included by this statement are genes which exclude non-coding regions, such as those shown in Figure 3, genes containing the individual structural genes coding for the signal peptide chain and the A, B and C chains of human preprorelaxin (see 35 Figure 3) and any combinations of these chains, e.g., the genes for expressing the A and B peptide chains, separately or as prorelaxin (with the C chain).

Thus according to another aspect of the present invention, there is provided a gene for the expression of human prorelaxin.

More specifically, this aspect of the invention provides a double-stranded DNA fragment for the expression of human prorelaxin, which comprises a coding strand and a complementary strand corresponding to the codons numbered as 1 to 160 of the mRNA sequence shown in Figure 2 of the 40 accompanying drawings.

According to a further aspect of the present invention, there are provided genes for the separate expression of the A, B and C chains of human relaxin or any combination of two or more of the said chains.

45 More specifically, this aspect of the invention provides double-stranded DNA fragments for the separate expression of the A and/or B and/or C chains of human relaxin, which comprise a coding strand and a complementary strand corresponding to the codons numbered 1 to 32, 33 to 136 and 137 to 160 of the mRNA sequence shown in Figure 2 of the accompanying drawings.

The genes described above in addition to the codons specified may also include the appropriate "start" 50 and "stop" codons, i.e., AUG and UGA respectively (codons -26 and 161 in Figure 2).

Those skilled in the art will appreciate that polymorphic forms of the genes may exist. Such forms are included in the present invention.

The invention further includes the complements of the above sequences, sub-units or equivalents, and the corresponding RNA sequences, sub-units or equivalents.

55 According to another aspect of the present invention there is provided a DNA transfer vector comprising the deoxynucleotide sequences corresponding to the genes defined above.

As shown above, the genetic code contains redundancies, that is certain amino acids are coded for by more than one codon. Thus the invention includes deoxynucleotide sequences in which the codons depicted in the drawings, or their cDNA equivalents are replaced by other codons which code for the same 60 amino-acid.

Furthermore, as already indicated above, peptides with relaxin activity may be produced which differ from the B and/or A chain structures of natural relaxin. Such differences may involve deletion of one or more amino acids and/or addition of further amino acids and/or substitution of different amino acids in the natural chains.

65 Thus the invention also includes genes and DNA transfer vectors as described above wherein one or

more of the natural codons are deleted and/or are replaced by codons which code for amino acids other than that coded by the natural codon, and/or further codons are added to the natural sequence.

The transfer vectors of the invention may also include *inter alia*, genetic information which ensures their replication when transferred to a host cell. Such cells may include, for example, the cells of prokaryotic microorganisms, such as bacteria, yeasts and moulds, and also eucaryotic cells, including mammalian cells and cell lines.

Examples of transfer vectors commonly used in bacterial genetics are plasmids and the DNA of certain bacteriophages. Both phage DNA and bacterial plasmids have been used as the transfer vectors in the present work. It will be understood however, that other types of transfer vectors may be employed. The general techniques of forming such transfer vectors and transforming them into microorganisms are well known in the art.

The invention also includes a prokaryotic or eukaryotic cell transformed by any of the transfer vectors described above.

One preferred microorganism is the very familiar *Escherichia coli*, but any other suitable microorganism may be used.

According to a still further aspect of the present invention, there is provided a process for making a DNA transfer vector for use in maintaining and replicating a deoxynucleotide sequence coding for human preprorelaxin, characterised by ligating a deoxynucleotide sequence coding for human preprorelaxin with a DNA molecule prepared by cleaving a transfer vector with a restriction enzyme.

DNA transfer vectors for use in maintaining and replicating deoxynucleotide sequences coding for human prorelaxin and for the A and B chains of human relaxin may be similarly prepared from the appropriate deoxynucleotides.

The A and B peptide chains, and also prorelaxin and preprorelaxin may be prepared by the usual process of gene expression, that is by growing cells containing the appropriate transformed transfer vector and isolating and purifying the required peptide(s) produced by the cells.

Thus, the invention further includes a process for making a fusion protein comprising the amino acid sequence of human preprorelaxin as its C-terminal sequence and a portion of a prokaryotic or eukaryotic protein as its N-terminal sequence, characterised by incubating a cell culture transformed by an expression transfer vector comprising a deoxynucleotide sequence coding for human preprorelaxin, prepared in accordance with the process described above.

Fusion proteins comprising the amino acid sequences for human prorelaxin and the A and B chains of human relaxin may be similarly prepared.

The fusion peptide products thus produced will be in the form of a fusion protein in which the desired peptide is linked with a portion of a prokaryotic or eukaryotic protein characteristic of the host cell. Such fusion proteins also form a part of this invention.

The invention also includes a process for synthesizing human prorelaxin comprising the A and B peptides separated from each other by a C peptide, characterised by incubating a culture of cells, transformed by an expression transfer vector comprising a deoxynucleotide sequence coding for said human prorelaxin, prepared as described above, under conditions suitable for expression of said sequence coding for human prorelaxin, and purifying human prorelaxin from the lysate or culture medium of said cells.

The peptide of interest can be recovered from the fusion product by any suitable known cleavage procedure.

As already indicated above the transfer vector may be modified by codon substitution/deletion/addition and such modifications will give rise to modified fusion peptides. In this way appropriate modifications may be made to facilitate the cleavage of the fusion peptides, for example, at the junction of B/C or C/A chains or to modify the peptide chain behaviour during subsequent chemical or biological processing.

As indicated above, the invention also provides human relaxin, prorelaxin and preprorelaxin.

Relaxin may be prepared by direct combination of the separate A and B chains by any of the procedures currently known and used for the preparation of insulin.

Also in a similar manner to insulin, relaxin may be prepared from prorelaxin by oxidizing or otherwise converting the sulfhydryl groups on the A and B peptides of relaxin, prepared as described herein, to form disulfide crosslinks between said A and B peptides, and then excising the C peptides, for example, by an enzyme-catalyzed hydrolysis specific for the bonds joining the C peptide to the A and B peptides.

The European patent application EP—A—287820, which is a divisional application of the present application, provides a method for the synthesis of human relaxin which comprises combining the A and B chains of relaxin (in their full-length, shortened or modified forms) by methods known *per se* for combination of A and B chains of human insulin.

One such method comprises reducing a mixture of the S-sulphonated A and B chains and then allowing the mixture to oxidize in air.

We have also found that the efficiency of the above procedure is improved when one or both of the A and B chains is in the form of an S-thioethyl-cys derivative rather than the S-sulpho form.

In our Australian Patent Application No. 15413/83 (PF 4385/82) we also showed that one or both of the A and B chains of relaxin can be shortened at the amino and/or carboxy terminii without significant loss of

biological activity and with improved combination yields. These techniques apply equally to the preparation of human relaxin.

The European patent application EP-A-287820 quoted above provides a human relaxin analogue consisting essentially of shortened and/or modified forms of the natural B and/or A peptide chains and also provides a method for producing a human relaxin analogue which comprises the step of forming the shortened and/or modified B and/or A peptide chains and combining them by any of the methods described above.

Our investigations with both pig and human relaxin show that relaxin activity may be present with A chains as short as A(10-24) and B chains as short as B(10-22) although the expected practical minima are respectively A(4-24) and B(4-23).

In general, the A chain can be varied from A(1-24) to A(10-24) and B chain from B(1-32) to B(10-22).

The preferred combinations are derived from:

15	A	B
	any of	(1-23)
	(1-24) (2-24) (3-24)	with any of (up to) (1-32)

20 Modifications of the B and/or A chains, in accordance with the present invention may involve either "genetic" modification, as described above or chemical modification of the B and/or A chains (in either full-length or shortened form) prior to combination by the methods of the invention. Two types of modification may be employed, either singly or in combination.

25 The first type involves the modification of one or more of the amino-acids which occur in the natural or shortened B and/or A chains. Such modification will generally involve protection of active groups on one or more of the amino-acids by methods known *per se*, and the protecting groups may, if desired, be removed after combination of the (modified) A and B chains.

30 Examples of this type of modification include the acetylation, formylation or similar protection of free amino groups, including the N-terminal, amidation of C-terminal groups, or the formation of esters of hydroxyl or carboxylic groups. The formyl group is a typical example of a readily-removable protecting group.

35 The second type of modification includes replacement of one or more of the natural amino-acids in the B and/or A chains with a different amino acid (including the D-form of a natural amino-acid). This general type of modification may also involve the deletion of a natural amino-acid from the chain or the addition of one or more extra amino-acids to the chain.

40 The purpose of such modifications is to enhance the combination yields of the A and B chains, while maintaining the activity of the product, i.e., relaxin or an analogue thereof, or to enhance or modify the activity of the product for a given combination yield. Such modification may extend to the production of synthetic analogues which have relaxin-blocking or -antagonistic effects.

45 A specific example of the first type of modification is the modification of the tryptophan (Trp) residue at B2 by addition of a formyl group.

50 Examples of the second type of modification are replacement of the Met moiety at B24 with norleucine (Nle), valine (Val), alanine (Ala), glycine (Gly), serine (Ser) or homoserine (HomoSer).

The invention in this aspect also includes human relaxin analogues formed from natural or shortened B and/or A chains modified in accordance with the invention as described above.

55 The A and B peptide chains, and also prorelaxin and preprorelaxin may be prepared by the usual process of gene expression, that is by growing a microorganism containing the appropriate transformed transfer vector and isolating and purifying the required peptide(s) produced by the microorganism.

The peptide products thus produced may be in the form of a fusion protein in which the desired peptide is linked with a portion of a prokaryotic protein.

60 The invention is further described and illustrated by the following description of the experimental procedures used and the results obtained thereby.

#### A. Experimental Procedures

##### 55 (i) Bacterial and Phage Strains

*E. coli* RR1 was used as the bacterial host for recombinant plasmids (pBR322) containing porcine relaxin cDNA insertions as described previously (7).

The library of human genomic clones was kindly provided by T. Maniatis. Genomic DNA fragments of about 15-20 kb, from the partial Hae 111/Alu 1 fragmentation of the human DNA (9), were cloned by ligation into the lambda phage vector Charon 4A (10) and propagated in *E. coli* LE392 cells.

65 Phage DNA (after clone selection) was prepared following lysis of *E. coli* DP50supF cells in 1 litre cultures (10).

Small DNA fragments (from fragmentation of phage DNA) were subcloned for sequence analysis into the M13 bacteriophage vectors mp7.1, mp8 and mp9 (kindly provided by Dr. J. Messing) and transformed into *E. coli* JM101 cells.

## (i) Preparation of hybridization probes (porcine DNA)

5 Radiolabelled probes were prepared by primed synthesis on various DNA fragments using denatured random primers (3 or 4 bases) of calf thymus DNA (11). The porcine DNA template (100—200 ng) was denatured with the random primers (1  $\mu$ g) by boiling in 20  $\mu$ l of H<sub>2</sub>O for 2 minutes. Synthesis was initiated by the addition of a 30  $\mu$ l reaction mixture containing 50mM Tris-HCl pH 8.0, 50mM NaCl, 1mM DTT, 10mM MgCl<sub>2</sub>, 5 units of *E. coli* DNA Polymerase 1, 500 M each of dCTP, dGTP, dTTP and 0.3  $\mu$ M  $\alpha$ -[<sup>32</sup>P]-dATP (approx. 3000 Ci/mmol, Amersham). After incubation at 37°C for 30 minutes the reaction was terminated by dilution into 300  $\mu$ l of a buffer containing 0.3M NaCl, 10mM Tris-HCl, pH 8.0, 1mM EDTA and passed through a Sephadex—G50 column, (1 cm x 5 cm) in the same buffer. The radiolabelled probe was collected 10 from the peak fractions at void volume and precipitated with 2 volumes of ethanol at -20°C for 2 hours using tRNA (10  $\mu$ g) as carrier.

## (iii) Screening procedures

15 Lambda phage ( $\lambda$ ) containing genomic DNA fragments were grown on soft agar at about 10<sup>5</sup> phage/13 cm diam. plate and transferred to nitrocellulose filters (Schleicher & Schull BA85) as described by Benton and Davis (12). Filters were hybridized with the radiolabelled probe at 40°C for 18 hours in modified Denhart's solution (13) containing 5  $\times$  SSC and 25% formamide. Filters were washed in 2  $\times$  SSC at 30° for 1 hour before exposing to x-ray film (Kodak XS—5) for 24 hours. Regions of the plate which exhibited positive hybridization were subcultured and rescreened successively until single positive plaques could be 20 selected. Phage were harvested after lysis of 1 litre cultures of *E. coli* I DP50supF cells and Df.A prepared by the methods described by Maniatis (10) and Yamamoto and Alberts (14).

## (iv) DNA Sequence Analysis

25 Restriction fragments of the selected recombinant phage were subcloned directly into the Eco R1, Pst 1 or Sma 1 site of phage M13mp8. Ligation were carried out in 20  $\mu$ l reactions containing 10mM Tris-HCl pH 8.0, 10mM MgCl<sub>2</sub>, 1mM DTT, 1mM ATP, 1 unit of T4 DNA ligase, DNA (100 ng) and the M13 phage vector (50 ng). After incubation at 40°C overnight recombinant DNA was transformed into *E. coli* JM101 cells (15). Plaques containing the coding region were selected by a similar technique as described for the genomic screens above, except the M13 phage were plated at lower density (10<sup>3</sup> phage/9 cm diam. plate). Positive 30 plaques were grown for a preparative yield of either single stranded template or replicative double stranded (rf) form (15). Single stranded templates were sequenced directly by the method of Sanger et al. (16) using either an M13-specific primer (Collaborative Research) or synthetic primers complementary to various sequences in the coding region. Complete sequence analysis of the subclones was obtained by cleavage of the rf form at several sites with various restriction enzymes followed by subcloning into M13 by 35 blunt end ligation (15) or by directly end-labelling fragments and sequencing by the method of Maxam and Gilbert (17). DNA sequence was analysed and compared to the porcine and rat relaxin sequences using computer programmes (18).

## B. Results

40 In the following discussion, reference will be made to the drawings. Figure 1 shows an abbreviated restriction enzyme map of the genomic clones.

45 Sizes are given in kilobase-pairs (kb) and cleavage sites are designated EcoR1 (R), Pst 1(P) and Hpa II (H). The genomic clone  $\lambda$ H5 terminates at an Eco R1 linker attached to the Alu 1 site in the C peptide (exon II) (A\* in Figure 1). The definitive nucleotide sequence over the coding region was compiled from the genomic clone  $\lambda$ H7 by subcloning Eco R1 and Pst 1 fragments into M13mp8 and then either:

- (1) direct sequencing shown by dashed lines in Figure 1 (---) on M13 templates
- (2) direct sequencing using synthetic nucleotide primers shown by dotted lines (....)
- (3) end-labelling DNA fragments and sequencing shown by solid lines (—) by chemical degradation.

50 The primers used for sequencing were

a: 5'TTCGCAATAGGCA and b: 5'GCACAAATTAGCT.

55 Figure 2 shows the coding region of the human relaxin gene.

A comparison of the human preprorelaxin amino acid and mRNA sequence (upper) with the corresponding porcine relaxin sequence (lower) is shown in Figure 3. The sequences have been aligned to maximize homology with nucleotide identities being indicated by asterisks and amino acid homologies by boxed-in areas. Amino acids are numbered from the start of the B-chain. The intron sequence at the exon/intron/exon boundaries is presented in lower case DNA notation.

## (ii) Isolation and characterization of genomic clones

60 Human genomic clones were identified by screening the library with probes made from a short (150bp) fragment of the porcine relaxin cDNA clone corresponding to amino acids 45—95 in the C-peptide (7) as set out in Figure 3 of the accompanying drawings. This fragment was excised from the clone by digestion with Hpa II and Hinfl and corresponded to the region of maximum homology (71% at the nucleotide level) between rat and porcine relaxin sequences. From the genomic clone bank, two strongly positive phage designated  $\lambda$ H5 and  $\lambda$ H7 were isolated. These positive clones were further characterized by restriction enzyme analysis using as probes two separate fragments of porcine relaxin cDNA specific for the 5' and 3' exon regions respectively (hereinafter called "exon I" and "exon II"). The two fragments were generated by

cleavage of the porcine relaxin cDNA clone at a single *Hpa* II site which corresponds (within a few bases) to an intron site in the homologous rat relaxin gene (6). Southern blot analysis of the  $\lambda$ H5 and  $\lambda$ H7 clones revealed that the coding region of the human relaxin gene is interrupted by a single intron of 3.4 kb (see Figure 1).

5 (ii) Sequence Analysis of the Genomic Clones

The strategy used was to subclone complete restriction digests of  $\lambda$ H5 and  $\lambda$ H7 into M13 vectors and then screen using porcine relaxin probes specific for exons I and II. The positive subclones were sequenced by a combination of techniques described in the methods section (A(iv) above).

10 The exon II region of the  $\lambda$ H7 clone was contained in a 2.0 kb Eco R1 fragment beginning at an Eco R1 site in the C-peptide and continuing through the entire coding sequence of the A chain to the termination codon (see Fig. 1). Sequencing of this fragment was aided considerably by the synthesis of nucleotide primers specific for regions around the A chain which were used to prime directly on the M13 template containing the entire 2.0 kb fragment. The subcloned Eco R1 fragment containing the remaining 53 bp of 15 the C-peptide in exon II could not be identified with the porcine cDNA as a probe. The sequence over this region was obtained by a subcloned Pst I fragment from  $\lambda$ H7 which contained the entire exon II region.

Sequencing the exon II region of  $\lambda$ H5 revealed an extremely short 70 bp fragment beginning at the same Eco R1 site in the C-peptide as  $\lambda$ H7 (see Fig. 1) but terminating with an Eco R1 linker which had been attached to an Alu I site in the original genomic DNA during the generation of the genomic library. Thus

20  $\lambda$ H5 was designated an incomplete clone of the relaxin gene and was not analysed further.

Sequence analysis of the exon I region was slightly complicated by an Eco R1 site in the signal peptide which necessitated the independent sequencing of two Eco R1 fragment subclones. The overlap over the Eco R1 site was supported by the identification of a Alu I subclone from  $\lambda$ H7 which contained the overlapping sequence.

25

C. Synthesis of a modified human relaxin (hRLX) A(1-24) — B(1-25)

(i) Synthesis of human relaxin A-chain, hRLX A(1-24)

The amino acid sequence corresponding to residues 1 to 24 of the human relaxin A-chain, deduced as described above from the nucleotide sequence of the genomic clone, was synthesized by the solid-phase procedure according to the general principles described by Merrifield (e.g. Barany, G. and Merrifield, R. B. In "The Peptides", Ed. E. Gross & J. Meienhofer, Academic Press, N.Y., pp. 1-284, 1980).

30 N- $\alpha$ -tertiarybutyloxycarbonyl\*-4-methylbenzyl-L-cysteine (\*hereinafter "BOC") was coupled to a 1% crosslinked polystyrene resin via the phenylacetamidomethyl (PAM) linkage to a level of 0.30 mmole/gm using the method of Tam et al., (Synthesis 12, 955-957, 1979). The BOC-L-CYS-PAM (8.0 gm) was 35 transferred to the reaction vessel of a Beckman Model 990 Peptide Synthesizer and the amino acid sequence from residues 23 through to 1 was assembled by the stepwise addition of each suitably protected amino acid. The amino terminal BOC protecting group of each amino acid was removed by treatment of the resin with 35% trifluoroacetic acid in methylene chloride for 30 minutes followed by neutralization with 5% diisopropylethylamine in methylene chloride for 15 minutes. After each treatment the resin was washed thoroughly with methylene chloride. The next amino acid in the sequence (suitably protected at the  $\alpha$ -amino with the BOC group and where necessary with the side-chain functional group appropriately protected) was coupled to the resin using dicyclohexylcarbodiimide (DCC). The resin was stirred with the amino acid in methylene chloride for 10 minutes prior to the introduction of the DCC which was also dissolved in methylene chloride. A 2.5 molar excess (6.0 mmole) of amino acid and DCC was used for each coupling. After stirring for 1 hour a sample of the resin was removed from the reaction mixture and tested for the presence of free amino groups using the ninhydrin procedure of Kaiser et al. (Anal. Biochem., 34, 595-598, 1970). If the ninhydrin test was negative indicating complete coupling the reaction cycle was continued with BOC deprotection, neutralization and coupling of the next amino acid. For a positive ninhydrin test the coupling reaction was repeated with further amino acid and DCC.

40 50 Amino acids with side-chain functional groups were used as the following protected derivatives: N- $\alpha$ -BOC-2,6-dichlorobenzyl-L-tyrosine, N- $\alpha$ -BOC- $\epsilon$ -chlorobenzylcarbonyl-L-lysine; N- $\alpha$ -BOC-L-serine O-benzyl ether; N- $\alpha$ -amylloxycarbonyl-N<sup>G</sup>-tosyl-L-arginine; N- $\alpha$ -BOC-L-threonine O-benzyl ether; N- $\alpha$ -BOC-S-ethyl mercapto-L-cysteine (for CYS at A-chain sequence position 15, 11 and 10); N- $\alpha$ -BOC-L-glutamic acid- $\gamma$ -benzyl ester.

55 Following the assembly of the 1-24 peptide sequence, the final BOC group on the amino terminal arginine was removed using the deprotection neutralization cycle and the peptide-resin dried in vacuo (wt of peptide resin 17.0 gm). A portion of the peptide-resin (2 gm) was treated with anhydrous hydrogen fluoride in the presence of anisole (2 ml) at 0°C for 30 minutes. The total time for contact of the resin-peptide with hydrogen fluoride (HF) was kept to a minimum (not more than 70 minutes) by rapid removal of the HF under oil-pump vacuum. The resin-peptide was then washed several times with ethyl acetate to remove excess anisole, the peptide extracted into 1M acetic acid and the solution lyophilized. The yield of crude peptide, (with the cysteines at positions 10, 11 and 15 still protected as the S-thioethyl derivative) was 440 mg. Initial purification of the crude peptide was by gel-filtration on Biogel P10 in 0.1M acetic acid. The fractions representing the major peak from this column, which eluted at a position corresponding to a molecular weight of approximately 3000, were collected and lyophilized. Amino acid analysis of a sample

of this peptide indicated that all the amino acids of the 1-24 sequence were present in the correct ratio. Further purification of the [S-thioethyl Cys<sup>10,11,15</sup>]-hRLX A(1-24) peptide was effected by preparative reverse-phase HPLC on a Waters C-18 Bondapak column using a 0.1% TFA-water/acetonitrile solvent system.

A sample (160 mg) of the peptide purified by gel-filtration was S-sulfonated with a mixture of sodium sulfite and sodium tetrathionate (total reaction time of 3 hours) according to the method described by Du et al., (Scientia Sinica, 101, 84-104 (1961)). The precipitate which formed during the S-sulfonation reaction was removed by filtration and both the precipitate and the supernatant solution dialyzed against distilled water at 4°C for 48 hours. The contents of the dialysis bags were lyophilized to yield 81.4 mg of peptide from the supernatant solution and 53.2 mg of peptide from the precipitate which occurred during the S-sulfonation reaction. A sample of the 'soluble' [S-sulfo Cys<sup>10,11,15,24</sup>] hRLX A(1-24) peptide was purified by ion exchange chromatography on DEAE-cellulose in tris-HCl buffer pH 8.3. Peptide was eluted from the column with a linear gradient of NaCl in tris-HCl buffer using a conductivity range of 3.0 mS to 85.0 mS. Fractions representing the major peak eluting from the ion-exchange column at conductivity 20 to 30 mS were dialyzed and the peptide recovered by lyophilization. Prepared HPLC was used to further purify the S-sulfonated peptide.

(ii) Synthesis of shortened human relaxin B-chain, hRLX B(1-25)

The amino acid sequence corresponding to residues 1 to 25 of the human relaxin B-chain was synthesized using the procedures described above and commencing with 7.0 gm N- $\alpha$ -tertiarybutyloxy-carbonyl-O-benzyl-L-serine-phenylacetamido-methyl polystyrene resin with a loading of 0.1 mmole Ser per gm. The side-chain protecting groups used in the A-chain synthesis were also employed for the B-chain including the S-ethyl derivative for both cysteines at positions 10 and 22. The aspartic acid residues at positions 4 and 5 were added as the N- $\alpha$ -BOC- $\epsilon$ -benzyl ester derivative. The glutamine at position 18 was coupled by the active ester procedure using N- $\alpha$ -BOC-L-glutamine-p-nitrophenyl ester in DMF. Following coupling of the tryptophan at position 2, 0.1% indole was added to the trifluoroacetic acid deprotecting reagent and to the subsequent methylene chloride washes.

The final weight of peptide-resin after removal of the BOC group from the amino terminal lysine residue and vacuum-drying was 12.2 gm. A portion of the peptide resin (5 gm) was treated with anhydrous hydrogen fluoride in the presence of anisole (2 ml) at 0°C for 30 minutes and the B-chain peptide isolated using the procedure described above for the A-chain. The crude [S-thioethyl Cys<sup>10,22</sup>] hRLX B(1-25) (1.40 gm) was purified by gel filtration on BioGel P10 in 0.1M acetic acid followed by preparative HPLC.

A sample (150 mg) of the peptide purified by gel filtration was S-sulfonated at pH 8.3 for 3 hours, the reaction mixture filtered and the precipitate and supernatant solutions dialyzed against distilled water. The 'soluble' peptide recovered after lyophilization was 92 mg; the 'insoluble' peptide was 55 mg. The S-sulfonated B-chain peptides were further purified by preparative HPLC using a C-18 reverse-phase column and 0.1% TFA-water-acetonitrile solvent system.

(iii) Chain Combination

The synthetic hRLX A(1-24) and hRLX B(1-25) peptides were combined using the procedure described by Chance and Hoffmann (Australian Patent Application No. 68844/81) for insulin chains wherein the S-sulfonated peptides were mixed in a ratio of A:B of 2:1 at a peptide concentration of 10 mg/ml in glycine buffer pH 10.5. Dithiothreitol in glycine buffer was then added in an amount to give a total of 1.0 sulphydryl groups for each S-sulfo group. The reaction mixture was then stirred in an open vessel for 24 hours.

As a further modification to this procedure we have found that the chain combination reaction to form biologically active relaxin proceeded efficiently when one or preferably both of the peptide chains are used as their S-thioethyl-Cys derivatives rather than in the S-sulfo form specified by Chance and Hoffmann (op. cit.) in the case of insulin. The use of S-thioethyl Cys peptides eliminates a reaction and purification step required to convert the peptides to the S-sulfo derivatives. In our experience the S-sulfonation reaction of relaxin peptides is accompanied by side reactions which render the S-sulfo relaxins difficult to purify resulting in low yields.

Using the above conditions chain combination yields from 0.24 to 3.1% have been achieved as measured by biological activity in the rat uterine contractility assay of Wiqvist & Paul (Acta Endocrinol., 29, 135-136, 1958).

Example of Chain Combination Reaction

Human relaxin [S-thioethyl Cys<sup>10,11,15</sup>] A(1-24) (3.60 mg dry wt., 2.0 mg peptide by amino acid analysis, 0.68  $\mu$ mole) was dissolved in 200  $\mu$ l of 0.1M glycine buffer pH 10.5 in a 3 ml stoppered plastic centrifuge tube. Human relaxin [S-sulfo Cys<sup>10,11</sup>] B(1-25) (1.89 mg, 1.0 mg peptide by amino acid analysis, 0.33  $\mu$ mole) dissolved in 100  $\mu$ l of 0.1M glycine buffer pH 10.5 was added and the mixture agitated. An aliquot (15.2  $\mu$ l, 1.73  $\mu$ mole DTT) of a stock solution of dithiothreitol (DTT) made up in 0.1 M glycine buffer pH 10.5 (1.15  $\mu$ mole DTT in 10 ml) was added to the peptide solution and following a brief agitation the reaction mixture was allowed to stand at 4°C for 24 hours open to the air. The mixture was then centrifuged and an aliquot of the supernatant solution tested for relaxin biological activity in the rat uterine contractility assay. Aliquots of the reaction mixture inhibited the spontaneous contractions of the rat uterus in a dose-

related manner. A 75  $\mu$ l aliquot completely inhibited uterine contractions equivalent to a chain combination yield of 0.70% as compared to a native pig relaxin A22 B31 standard.

5 Additional synthetic human relaxin peptides based upon the H1-gene sequence:

The synthetic relaxin peptides listed in the following table were prepared from the amino acid sequences for the A and B chains derived from the H1 human relaxin gene sequence shown in Figure 2. The separate peptide chains were prepared and purified according to the procedure described above for the A(1-24) and B(1-25) peptides. A modification of these procedures was used for the B(3-25) amide and B(1-25) amide peptides, wherein the PAM resin linkage was replaced by the benzhydrylamine (BHA) polystyrene resin. Use of the BHA resin results in the formation of peptides with the C-terminus in the amide rather than free carboxy form.

10 Unless otherwise stated the chain combination reaction was performed as described previously with the A-chain as the S-thioethyl Cys derivative and the B-chain as the S-sulfo Cys derivative.

15 All of the synthetic analogues in the following table exhibited relaxin-like biological activity in the rat uterine contractility assay. The combination yields of the separate peptide chains were calculated from the bioassay results using native pig relaxin A(1-22)-B(1-31) as standard.

TABLE

	Synthetic H1 Human Relaxin Analogue	Combination Yield (based on B-chain amount)
25	A(1-24) + B(1-23)	0.24%
	A(1-24) + B(1-25)	0.70%
30	A(1-24) + [Ala <sup>24</sup> ]B(1-26)	0.92%
	A(1-24) + B(1-32)	2.00%
35	A(1-24) + B(1-25) amide	0.80%
	A(1-24) + B(1-25) amide with both chains in S-thioethyl form for chain combination reaction	3.10%
40	A(1-24) + B(3-25) amide	0.68%
	A(1-24) + [N-formyl Trp <sup>2</sup> ]B(2-25)	0.43%

45 The features disclosed in the foregoing description, in the following claims and/or in the accompanying drawings may, both separately and in any combination thereof, be material for realising the invention in diverse forms thereof.

## References

1. Hisaw, F. L. Proc. Soc. Exp. Biol. Med. 23, 661-663 (1926).
2. Schwabe, C., McDonald, J. K. and Steinetz, B. C. Biochem. Biophys. Res. Commun. 75, 503-510 (1977).
3. James, R., Niall, H., Kwok, S. and Bryant-Greenwood, G. Nature, 267, 544-546 (1977).
4. John, M. J., Walsh, J. R., Borjesson, B. W. and Niall, H. D. Endocrinology 108, 726-729 (1981).
5. Schwabe, C., Gowan, L. K. and Reinig, J. W. Ann. N.Y. Acad. Sci. 380, 8-12 (1982).
6. Hudson, P., Haley, J., Cronk, M., Shine, J. and Niall, H. Nature, 291, 127-131 (1981).
7. Haley, J., Hudson, P., Scanlon, D., John, M., Cronk, M., Shine, J., Tregear, G. and Niall, H. DNA 1, 155-162 (1982).
8. Dayhoff, M. O., Schwartz, R. M., Chen, H. R., Hunt, L. T., Barker, W. C. and Orcutt, B. C. DNA 1, 51-58 (1981).
9. Lawn, R. M., Fritsch, E. F., Parker, R. C., Blake, G. and Maniatis, T. Cell, 15, 1157-1174 (1978).
10. Maniatis T., Hardison, R. E., Lacy, E., Lauer, J., O'Connell, C., and Quon, D. Cell 15, 687-701 (1978).
11. Taylor, J. M., Illmersee, R., and Summers, J. Biochim. Biophys. Acta 442, 324-330 (1976).
12. Benton, W. D. and Davis, R. Science 196, 180-183 (1977).
13. Denhardt, D. T. Biochem. Biophys. Res. Commun. 23, 641-646 (1966).
14. Yamamoto, K. R. and Alberts, B. M. Virology 40, 734-744 (1970).

## References — continued

5 15. Sanger, F., Coulson, A. R., Barrell, B. G., Smith A. J. A. and Roe, B. A. J. Mol. Biol. 143, 161—178 (1980).  
 16. Sanger, F., Nicklen, S. and Coulson, A. R. Proc. Natn. Acad. Sci. 74, 5463—5467 (1977).  
 17. Maxam, A. M. and Gilbert, W. Proc. Natn. Acad. Sci. 74, 560—564 (1977).  
 18. Staden, R. Nucl. Acids, Res. 6, 2601—2610 (1979).

## Claims

10 1. A DNA fragment encoding human H1-preprorelaxin, characterized in that it comprises a coding strand and a complementary strand corresponding to the following complete mRNA sequence:

15 AUG CCU CGC CUG UUC UUG UUC CAC CUG CUA GAA UUC UGU UUA  
 20 CUA CUG AAC CAA UUU UCC AGA GCA GUC GCG GCC AAA UGG AAG  
 25 GAC GAA GUU AUU AAA UUA UGC GGC CGC GAA UUA GUU CGC GCG  
 30 CAG AUU GCC AUU UGC GGC AUG AGC ACC UGG AGC AAA AGG UCU  
 35 CUG AGC CAG GAA GAA GCU CCU CAG ACA CCU AGA CCA GUG GCA  
 AUU AUC AUG UUG GAA UUC AUU GCU AAU UUG CCA CCG GAG CUG  
 AAG GCA GCC CUA UCU GAG AGG CAA CCA UCA UUA CCA GAG CUA  
 CAG CAG UAU GUA CCU GCA UUA AAG GAA UCC AAU CUU AGC UUU  
 GAA GAA UUU AAG AAA CUU AUU CGC AAU AGG CAA AGU GAA GCC  
 GCA GAC AGC AAU CCU UCA GAA UUA AAA UAC UUA GGC UUG GAA  
 ACU CAU UCU CAA AAA AAG AGA CGA CCC UAC GUG GCA CUG UUU  
 GAG AAA UGU UGC CUA AUU GGU UGU ACC AAA AGG UCU CUU GCU  
 AAA UAU UGC UGA

35 2. A DNA fragment encoding human H1-prorelaxin, characterized in that it comprises a coding strand and a complementary strand corresponding to the following complete mRNA sequence:

40 AAA UGG AAG GAC GAA GUU AUU AAA UUA UGC GGC CGC GAA UUA  
 45 GUU CGC GCG CAG AUU GCC AUU UGC GGC AUG AGC ACC UGG AGC  
 CCA GUG GCA GAA AUU GUA CCA UCC UUC AUC AAC AAA GAA ACA  
 GAA ACU AUU AUU AUC AUG UUG GAA UUC AUU GCU AAU UUG CCA  
 CCG GAG CUG AAG GCA GCC CUA UCU GAG AGG CAA CCA UCA UUA  
 CCA GAG CUA CAG CAG UAU GUA CCU GCA UUA AAG GAA UCC AAU  
 CUU AGC UUU GAA GAA UUU AAG AAA CUU AUU CGC AAU AGG CAA  
 AGU GAA GCC GCA GAC AGC AAU CCU UCA GAA UUA AAA UAC UUA  
 GGC UUG GAA ACU CAU UCU CAA AAA AAG AGA CGA CCC UAC GUG  
 GCA CUG UUU GAG AAA UGU UGC CUA AUU GGU UGU ACC AAA AGG  
 UCU CUU GCU AAA UAU UGC UGA

55 60 3. A DNA fragment as claimed in Claim 2, which is a double-stranded DNA fragment encoding the signal, A, B or C peptide chains of human H1-preprorelaxin or a combination of any two or more of said chains characterized in that it comprises a coding strand and a complementary strand corresponding to the appropriate mRNA sequence or combination of the mRNA sequences given below:

Signal Peptide

5 AUG CCU CGC CUG UUC UUG UUC CAC CUG CUA GAA UUC UGU UUA  
 CUA CUG AAC CAA UUU UCC AGA GCA GUC GCG GCC

A-Chain

10 CGA CCC UAC GUG GCA CUG UUU GAG AAA UGU UGC CUA AUU GGU  
 UGU ACC AAA AGG UCU CUU GCU AAA UAU UGC

B-Chain

15 AAA UGG AAG GAC GAU GUU AUU AAA UUA UGC GGC CGC GAA UUA  
 GUU CGC GCG CAG AUU GCC AUU UGC GGC AUG AGC ACC UGG AGC  
 AAA AGG UCU CUG

C-Chain

20 AGC CAG GAA GAU GCU CCA CAG ACA CCA GUG GCA GAA  
 AUU GUA CCA UCC UUC AUC AAC AAA GAU ACA GAA ACU AUA AUU  
 AUC AUG UUG GAA UUC AUU GCU AAU UUG CCA CCG GAG CUG AAG  
 GCA GCC CUA UCU GAG AGG CAA CCA UCA UUA CCA GAG CUA CAG  
 CAG UAU GUA CCU GCA UUA AAG GAU UCC AAU CUU AGC UUU GAA  
 GAA UUU AAG AAA CUU AUU CGC AAU AGG CAA AGU GAA GCC GCA  
 GAC AGC AAU CCU UCA GAA UUA AAA UAC UUA GGC UUG GAU ACU  
 CAU UCU CAA AAA AAG AGA

35 4. A process for the production of a DNA fragment as set out in any one of Claims 1 to 3, characterized in that it comprises screening a human genomic clone bank using as a probe a fragment of porcine relaxin cDNA having the following nucleotide sequence which corresponds to amino acids 45-95 in the C-peptide thereof:

40 GCA GAA ACC ATG CCA TCC TCC ATC ACC AAA GAT GCA GAA ATC  
 TTA AAG ATG ATG TTG GAA TTT GTT CCT AAT TTG CCA CAG GAG  
 CTG AAG GCA ACA TTG TCT GAG AGG CAA CCA TCA CTG AGA GAG  
 CTA CAA CAA TCT GCA TCA AAG GAT TCG.

45 5. A DNA transfer vector, characterized in that it contains a cDNA deoxynucleotide sequences corresponding to a DNA fragment as defined in any one of Claims 1 to 3.

6. A DNA fragment or DNA transfer vector as claimed in any of Claims 1 to 3 and 5, characterized in that one or more natural codons or their cDNA equivalents of said DNA fragments are replaced by another codon which codes for the same amino acid.

50 7. A DNA transfer vector as claimed in any one of Claims 5 or 6, characterized in that it is a bacterial plasmid.

8. A DNA transfer vector as claimed in any one of Claims 5 to 7 which is bacteriophage DNA.

9. A cell transformed by a transfer vector as claimed in any one of Claims 5 to 8.

10. A process for making a DNA transfer vector for use in maintaining and replicating a DNA fragment as claimed in any one of claims 1 to 3 and 6, characterized in that it comprises reacting the appropriate deoxynucleotide sequence of the said DNA fragment with a DNA molecule prepared by cleaving a transfer vector with a restriction enzyme.

60 11. A process for making a fusion protein comprising an amino acid sequence consisting of all or part of the amino acid sequence of human H1-preprorelaxin as its C-terminal sequence and a portion of a prokaryotic protein as its N-terminal sequence, characterized in that it comprises incubating a microorganism transformed by an expression transfer vector comprising the appropriate deoxynucleotide sequence.

65 12. A process for synthesizing human H1-prorelaxin comprising the A and B peptides separated from each other by a C peptide, characterized in that it comprises incubating a microorganism, transformed by an

expression transfer vector comprising a DNA fragment coding for said human prorelaxin as claimed in Claim 2 under conditions suitable for expression of said DNA fragment, and purifying human prorelaxin from the lysate or culture medium of said microorganism.

13. A polypeptide with human H1-preprorelaxin activity, said polypeptide being selected from the following group:

- 5 (a) a polypeptide having the amino acid sequence shown in Figure 2 A and B that corresponds to human H1-preprorelaxin;
- (b) a polypeptide that in respect to (a) is deficient in one or more amino acids;
- 10 (c) a polypeptide in which in respect to (a) one or more amino acids are replaced with different amino acids;
- (d) a polypeptide in which in respect to (a) one or more amino acids are added to the sequence; or
- (e) a fusion polypeptide comprising an amino acid sequence consisting of all or part of the amino acid sequence of human H1-preprorelaxin shown in Figure 2 A and B as its C-terminal sequence and a portion of a prokaryotic protein as its N-terminal sequence.

15 14. A polypeptide with human H1-prorelaxin activity, said polypeptide being selected from the following group:

- 5 (a) a polypeptide having the amino acid sequence shown in Figure 2 A and B that corresponds to human H1-prorelaxin;
- (b) a polypeptide that in respect to (a) is deficient in one or more amino acids;
- 10 (c) a polypeptide in which in respect to (a) one or more amino acids are replaced with different amino acids;
- (d) a polypeptide in which in respect to (a) one or more amino acids are added to the sequence; or
- (e) a fusion polypeptide comprising an amino acid sequence consisting of all or part of the amino acid sequence of human H1-prorelaxin shown in Figure 2 A and B as its C-terminal sequence and a portion of a prokaryotic protein as its N-terminal sequence.

20 15. A polypeptide with human H1-relaxin activity, said polypeptide being selected from the following group:

- 5 (a) a polypeptide having the amino acid sequence shown in Figure 2 A and B that corresponds to human H1-relaxin except that it is deficient in one or more amino acids;
- (b) a polypeptide having the amino acid sequence shown in Figure 2 A and B that corresponds to human H1-relaxin except that one or more amino acids are replaced with different amino acids;
- 10 (c) a polypeptide having the amino acid sequence shown in Figure 2 A and B that corresponds to human H1-relaxin except that one or more amino acids are added to the sequence; or
- (d) a fusion polypeptide comprising an amino acid sequence consisting of all or part of the amino acid sequence of human H1-relaxin shown in Figure 2 A and B as its C-terminal sequence and a portion of a prokaryotic protein as its N-terminal sequence.

25 16. A process for the isolation of a chromosomal relaxin gene encoding a human H1-relaxin according to claim 15, characterized in that it comprises screening a genomic library using as a probe a sub-unit of the porcine relaxin cDNA.

30 17. An isolated DNA sequence comprising a DNA sequence encoding human H1-preprorelaxin, human H1-prorelaxin, or human H1-relaxin which has been modified by deletion of one or more of the natural codons, to encode a peptide comprising an A chain selected from the group consisting of A chain amino acids 1-24 to 3-24 and a B chain selected from the group consisting of B chain amino acids 1-32 to 4-23, said peptide having human H1-relaxin activity.

35 18. An isolated DNA sequence comprising a DNA sequence encoding human H1-preprorelaxin, human H1-prorelaxin or human H1-relaxin which has been modified by replacement of one or more of the natural codons by codons which code for amino acids other than that coded by the natural codon, to encode a peptide comprising an A chain selected from the group consisting of A chain amino acids 1-24 to 3-24 and a B chain selected from the group consisting of B chain amino acids 1-32 to 4-23, wherein the Met 24 of the B chain is substituted with Ala, Val, Gly or Ser, said peptide having human H1-relaxin activity.

40 19. A DNA fragment encoding a polypeptide with human H1-preprorelaxin activity, said polypeptide being selected from the following group:

- 5 (a) a polypeptide having the amino acid sequence shown in Figure 2 A and B that corresponds to human H1-preprorelaxin;
- (b) a polypeptide that in respect to (a) is deficient in one or more amino acids;
- 10 (c) a polypeptide in which in respect to (a) one or more amino acids are replaced with different amino acids;
- (d) a polypeptide in which in respect to (a) one or more amino acids are added to the sequence; or
- (e) a fusion polypeptide comprising an amino acid sequence consisting of all or part of the amino acid sequence of human H1-preprorelaxin shown in Figure 2 A and B as its C-terminal sequence and a portion of a prokaryotic protein as its N-terminal sequence.

45 20. A DNA fragment encoding a polypeptide with human H1-prorelaxin activity, said polypeptide being selected from the following group:

- 5 (a) a polypeptide having the amino acid sequence shown in Figure 2 A and B that corresponds to human H1-prorelaxin;

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(b) a polypeptide that in respect to (a) is deficient in one or more amino acids;

5 (c) a polypeptide in which in respect to (a) one or more amino acids are replaced with different amino acids;

(d) a polypeptide in which in respect to (a) one or more amino acids are added to the sequence; or

10 (e) a fusion polypeptide comprising an amino acid sequence consisting of all or part of the amino acid sequence of human H1-prorelaxin shown in Figure 2 A and B as its C-terminal sequence and a portion of a prokaryotic protein as its N-terminal sequence.

15 21. An isolated DNA fragment encoding a polypeptide with human H1-relaxin activity, said polypeptide being selected from the following group:

10 (a) a polypeptide having the amino acid sequence shown in Figure 2 A and B that corresponds to human H1-relaxin;

(b) a polypeptide that in respect to (a) is deficient in one or more amino acids;

15 (c) a polypeptide in which in respect to (a) one or more amino acids are replaced with different amino acids;

(d) a polypeptide in which in respect to (a) one or more amino acids are added to the sequence; or

20 (e) a fusion polypeptide comprising an amino acid sequence consisting of all or part of the amino acid sequence of human H1-relaxin shown in Figure 2 A and B as its C-terminal sequence and a portion of a prokaryotic protein as its N-terminal sequence.

20 Patentansprüche

1. DNA-Fragment, das menschliches H1-Präprorelaxin codiert dadurch gekennzeichnet, daß es einen codierenden Strang und einen komplementären Strang, entsprechend der folgenden vollständigen mRNA-Sequenz, umfaßt:

25 AUG CCU CGC CUG UUC UUG UUC CAC CUG CUA GAA UUC UGU UUA  
CUA CUG AAC CAA UUU UCC AGA GCA GUC GCG GCC AAA UGG AAG  
GAC GAU GUU AUU AAA UUA UGC GGC CGC GAA UUA GUU CGC GCG  
30 CAG AUU GCC AUU UGC GGC AUG AGC ACC UGG AGC AAA AGG UCU  
CUG AGC CAG GAA GAU GCU CCU CAG ACA CCU AGA CCA GUG GCA  
GAA AUU GUA CCA UCC UUC AUC AAC AAA GAU ACA GAA ACU AUA  
35 AUU AUC AUG UUG GAA UUC AUU GCU AAU UUG CCA CCG GAG CUG  
AAG GCA GCC CUA UCU GAG AGG CAA CCA UCA UUA CCA GAG CUA  
CAG CAG UAU GUA CCU GCA UUA AAG GAU UCC AAU CUU AGC UUU  
40 GAA GAA UUU AAG AAA CUU AUU CGC AAU AGG CAA AGU GAA GCC  
GCA GAC AGC AAU CCU UCA GAA UUA AAA UAC UUA GGC UUG GAU  
ACU CAU UCU CAA AAA AAG AGA CGA CCC UAC GUG GCA CUG UUU  
45 GAG AAA UGU UGC CUA AUU GGU UGU ACC AAA AGG UCU CUU GCU  
AAA UAU UGC UGA

50 2. DNA-Fragment, das menschliches H1-Prorelaxin codiert, dadurch gekennzeichnet, daß es einen codierenden Strang und einen komplementären Strang, entsprechend der folgenden mRNA-Sequenz, umfaßt:

55 AAA UGG AAG GAC GAU GUU AUU AAA UUA UGC GGC CGC GAA UUA  
GUU CGC GCG CAG AUU GCC AUU UGC GGC AUG AGC ACC UGG AGC  
AAA AGG UCU CUG AGC CAG GAA GAU GCU CCU CAG ACA CCU AGA  
CCA GUG GCA GAA AUU GUA CCA UCC UUC AUC AAC AAA GAU ACA  
GAA ACU AUA AUU AUC AUG UUG GAA UUC AUU GCU AAU UUG CCA  
60 CCG GAG CUG AAG GCA GCC CUA UCU GAG AGG CAA CCA UCA UUA  
CCA GAG CUA CAG CAG UAU GUA CCU GCA UUA AAG GAU UCC AAU  
CUU AGC UUU GAA GAA UUU AAG AAA CUU AUU CGC AAU AGG CAA  
AGU GAA GCC GCA GAC AGC AAU CCU UCA GAA UUA AAA UAC UUA

5 GGC UUG GAU ACU CAU UCU CAA AAA AAG AGA CGA CCC UAC GUG  
 10 GCA CUG UUU GAG AAA UGU UGC CUA AUU GGU UGU ACC AAA AGG  
 UCU CUU GCU AAA UAU UGC UGA

15 3. DNA-Fragment nach Anspruch 2, das ein doppelsträngiges DNA-Fragment ist, welches die Signal-, A-, B- oder C-Peptidketten von menschlichem H1-Präprorelaxin oder eine Kombination von jeweils zwei oder mehr besagter Ketten codiert, dadurch gekennzeichnet, daß es einen codierenden Strang und einen komplementären Strang, entsprechend der passenden mRNA-Sequenz oder der Kombination der mRNA-Sequenzen, die unten angegeben sind, umfaßt:

20 Signalpeptid

25 AUG CCU CGC CUG UUC UUG UUC CAC CUG CUA GAA UUC UGU UUA  
 CUA CUG AAC CAA UUU UCC AGA GCA GUC GCG GCC

30 A-Kette

35 CGA CCC UAC GUG GCA CUG UUU GAG AAA UGU UGC CUA AUU GGU  
 UGU ACC AAA AGG UCU CUU GCU AAA UAU UGC

40 B-Kette

45 AAA UGG AAG GAC GAU GUU AUU AAA UUA UGC GGC CGC GAA UUA  
 GUU CGC GCG CAG AUU GCC AUU UGC GGC AUG AGC ACC UGG AGC  
 AAA AGG UCU CUG

50 C-Kette

55 AGC CAG GAA GAU GCU CCU CAG ACA CCU AGA CCA GUG GCA GAA  
 AUU GUA CCA UCC UUC AUC AAC AAA GAU ACA GAA ACU AUU AUU  
 AUC AUG UUG GAA UUC AUU GCU AAU UUG CCA CCG GAG CUG AAG  
 GCA GCC CUA UCU GAG AGG CAA CCA UCA UUA CCA GAG CUA CAG  
 CAG UAU GUA CCU GCA UUA AAG GAU UCC AAU CUU AGC UUU GAA  
 GAA UUU AAG AAA CUU AUU CGC AAU AGG CAA AGU GAA GCC GCA  
 GAC AGC AAU CCU UCA GAA UUA AAA UAC UUA GGC UUG GAU ACU  
 CAU UCU CAA AAA AAG AGA

60 4. Verfahren zur Herstellung eines DNA-Fragments nach einem der Ansprüche 1 bis 3, dadurch gekennzeichnet, daß es das Screenen einer menschlichen Genom-Klonbank unter Verwendung eines Fragments von Schweine-Relaxin-cDNA mit der folgenden Nukleotidsequenz, die den Aminosäuren 45—95 im C-Peptid desselben entspricht, als Sonde umfaßt:

65 GCA GAA ACC ATG CCA TCC TCC ATC ACC AAA GAT GCA GAA ATC  
 TTA AAG ATG ATG TTG GAA TTT GTT CCT AAT TTG CCA CAG GAG  
 CTG AAG GCA ACA TTG TCT GAG AGG CAA CCA TCA CTG AGA GAG  
 CTA CAA CAA TCT GCA TCA AAG GAT TCG.

70 5. DNA-Transfervektor, dadurch gekennzeichnet, daß er eine cDNA-Desoxynukleotidsequenz enthält, entsprechend einem DNA-Fragment, wie in einem der Ansprüche 1 bis 3 definiert.

75 6. DNA-Fragment oder DNA-Transfervektor nach einem der Ansprüche 1 bis 3 und 5, dadurch gekennzeichnet, daß ein oder mehrere natürliche Codons oder deren cDNA-Äquivalente besagter DNA-Fragment durch ein anderes Codon ersetzt sind, welches dieselbe Aminosäure codiert.

80 7. DNA-Transfervektor nach einem der Ansprüche 5 oder 6, dadurch gekennzeichnet, daß er ein bakterielles Plasmid ist.

85 8. DNA-Transfervektor nach einem der Ansprüche 5 bis 7, der Bakteriophagen-DNA ist.

90 9. Zelle, transformiert mit einem Transfervektor nach einem der Ansprüche 5 bis 8.

95 10. Verfahren zur Herstellung eines DNA-Transfervektors zur Verwendung bei der Aufrechterhaltung und Replikation eines DNA-Fragments nach einem der Ansprüche 1 bis 3 und 6, dadurch gekennzeichnet,

daß es das Umsetzen der passenden Desoxynukleotidsequenz des besagten DNA-Fragments mit einem DNA-Molekùl umfaßt, das durch Schneiden eines Transfervektors mit einem Restriktionsenzym hergestellt worden ist.

11. Verfahren zur Herstellung eines Fusionsproteins, das eine Aminosäuresequenz umfaßt, die 5 insgesamt oder teilweise aus einer Aminosäuresequenz von menschlichem H1-Präprorelaxin als seiner C-terminalen Sequenz und eines Teils eines prokaryontischen Proteins als seiner N-terminalen Sequenz besteht, dadurch gekennzeichnet, daß es das Inkubieren eines Mikroorganismus umfaßt, der mit einem Expressions-Transfervektor transformiert ist, welcher die passende Desoxynukleotidsequenz umfaßt.

12. Verfahren zu Synthese von menschlichem H1-Prorelaxin, welches die A- und B-Peptide, getrennt 10 voneinander durch ein C-Peptid, umfaßt, dadurch gekennzeichnet, daß es das Inkubieren eines Mikroorganismus, transformiert mit einem Expressions-Transfervektor, der ein DNA-Fragment umfaßt, das für besagtes menschliches Prorelaxin nach Anspruch 2 kodiert, unter für die Expression besagten DNA-Fragments geeigneten Bedingungen, und das Reinigen menschlichen Prorelaxins aus dem Lysat oder Kulturmedium von besagtem Mikroorganismus umfaßt.

13. Polypeptid mit der Aktivität von menschlichem H1-Präprorelaxin, wobei besagtes Polypeptid 15 ausgewählt ist aus der folgenden Gruppe:

- (a) ein Polypeptid mit der in Figur 2 A und B dargestellten Aminosäuresequenz, die menschlichem H1-Präprorelaxin entspricht;
- (b) ein Polypeptid, dem im Hinblick auf (a) eine oder mehrere Aminosäuren fehlen;
- 20 (c) ein Polypeptid, in dem im Hinblick auf (a) eine oder mehrere Aminosäuren durch andere Aminosäuren ersetzt sind;
- (d) ein Polypeptid, in dem im Hinblick auf (a) eine oder mehrere Aminosäuren zur Sequenz hinzugefügt sind; oder
- (e) ein Fusions-Polypeptid, das eine Aminosäuresequenz umfaßt, die insgesamt oder teilweise aus der 25 in Figur 2 A und B dargestellten Aminosäuresequenz von menschlichem H1-Präprorelaxin als seiner C-terminalen Sequenz und einem Teil eines prokaryontischen Proteins als seiner N-terminalen Sequenz besteht.

14. Polypeptid mit der Aktivität von menschlichem H1-Prorelaxin, wobei besagtes Polypeptid 30 ausgewählt ist aus der folgenden Gruppe:

- (a) ein Polypeptid mit der in Figur 2 A und B dargestellten Aminosäuresequenz, die menschlichem H1-Prorelaxin entspricht;
- (b) ein Polypeptid, dem im Hinblick auf (a) eine oder mehrere Aminosäuren fehlen;
- 35 (c) ein Polypeptid, in dem im Hinblick auf (a) eine oder mehrere Aminosäuren durch andere Aminosäuren ersetzt sind;
- (d) ein Polypeptid, in dem im Hinblick auf (a) eine oder mehrere Aminosäuren zur Sequenz hinzugefügt sind; oder
- (e) ein Fusions-Polypeptid, das eine Aminosäuresequenz umfaßt, die insgesamt oder teilweise aus der 40 in Figur 2 A und B dargestellten Aminosäuresequenz von menschlichem H1-Prorelaxin als seiner C-terminalen Sequenz und einem Teil eines prokaryontischen Proteins als seiner N-terminalen Sequenz besteht.

15. Polypeptid mit der Aktivität von menschlichem H1-Relaxin, wobei besagtes Polypeptid ausgewählt 45 ist aus der folgenden Gruppe:

- (a) ein Polypeptid mit der in Figur 2 A und B dargestellten Aminosäuresequenz, die menschlichem H1-Relaxin entspricht, mit der Ausnahme, daß ihm eine oder mehrere Aminosäuren fehlen;
- (b) ein Polypeptid mit der in Figur 2 A und B dargestellten Aminosäuresequenz, die menschlichem H1-Relaxin entspricht, mit der Ausnahme, daß eine oder mehrere Aminosäuren durch andere Aminosäuren 50 ersetzt sind;
- (c) ein Polypeptid mit der in Figur 2 A und B dargestellten Aminosäuresequenz, die menschlichen H1-Relaxin entspricht, mit der Ausnahme, daß eine oder mehrere Aminosäuren zur Sequenz hinzugefügt sind;
- (d) ein Fusions-Polypeptid, daß eine Aminosäuresequenz umfaßt, die insgesamt oder teilweise aus der 55 in Figur 2 A und B dargestellten Aminosäuresequenz von menschlichem H1-Relaxin als seiner C-terminalen Sequenz und einem Teil eines prokaryontischen Proteins als seiner N-terminalen Sequenz besteht.

16. Verfahren zur Isolierung eines chromosomalen Relaxin-Gens das ein menschliches H1-Relaxin 60 nach Anspruch 15 codiert, dadurch gekennzeichnet, daß es das Screenen einer Genombibliothek unter Verwendung einer Untereinheit der Schweine-Relaxin-cDNA als Sonde umfaßt.

17. Isolierte DNA-Sequenz, die eine DNA-Sequenz umfaßt, welche menschliches H1-Präprorelaxin, menschliches H1-Prorelaxin oder menschliches H1-Relaxin codiert, das durch Deletion einer oder mehrerer natürlicher Codons modifiziert worden ist, um ein Peptid zu codieren, das eine A-Kette, die ausgewählt ist 65 aus der Gruppe, die aus der A-Ketten-Aminosäuren 1—24 bis 3—24 besteht, und eine B-Kette, die ausgewählt ist aus der Gruppe, die aus B-Ketten-Aminosäuren 1—32 bis 4—23 besteht, umfaßt, wobei besagtes Peptid die Aktivität von menschlichem H1-Relaxin besitzt.

18. Isolierte DNA-Sequenz, die eine DNA-Sequenz umfaßt, die menschliches H1-Präprorelaxin, menschliches H1-Prorelaxin oder menschliches H1-Relaxin codiert, das durch Ersetzen einer oder mehrerer natürlicher Codons durch Codons, die für andere Aminosäuren codieren als für diejenigen, die durch das

natürliche Codons codiert werden, modifiziert worden sind, um ein Peptid zu codieren, das eine A-Kette, die ausgewählt ist aus der Gruppe, die aus A-Ketten-Aminosäuren 1—24 bis 3—24 besteht, und eine B-Kette, die ausgewählt ist aus der Gruppe, die aus B-Ketten-Aminosäuren 1—32 bis 4—23 besteht, umfaßt, wobei das Met 24 der B-Kette durch Ala, Val, Gly oder Ser ersetzt ist, wobei besagtes Peptid die Aktivität von

5 menschlichem H1-Relaxin besitzt.

19. DNA-Fragment, das ein Polypeptid mit der Aktivität von menschlichem H1-Präprorelaxin codiert, wobei besagtes Polypeptid ausgewählt ist aus der folgenden Gruppe:

(a) ein Polypeptid mit der in Figur 2 A und B dargestellten Aminosäuresequenz, die menschlichem H1-Präprorelaxin entspricht;

10 (b) ein Polypeptid, dem im Hinblick auf (a) eine oder mehrere Aminosäuren fehlen;

(c) ein Polypeptid, in dem im Hinblick auf (a) eine oder mehrere Aminosäuren durch andere Aminosäuren ersetzt sind;

(d) ein Polypeptid, in dem im Hinblick auf (a) eine oder mehrere Aminosäuren zur Sequenz hinzugefügt sind; oder

15 (e) ein Fusions-Polypeptid, das eine Aminosäuresequenz umfaßt, die insgesamt oder teilweise aus der in Figur 2 A und B dargestellten Aminosäuresequenz von menschlichem H1-Präprorelaxin als seiner C-terminalen Sequenz und einem Teil eines prokaryontischen Proteins als seiner N-terminalen Sequenz besteht.

20. DNA-Fragment, das ein Polypeptid mit der Aktivität von menschlichem H1-Prorelaxin codiert, wobei besagtes Polypeptid ausgewählt ist aus der folgenden Gruppe:

(a) ein Polypeptid mit der in Figur 2 A und B dargestellten Aminosäuresequenz, die menschlichem H1-Prorelaxin entspricht;

(b) ein Polypeptid, dem im Hinblick auf (a) eine oder mehrere Aminosäuren fehlen;

(c) ein Polypeptid, in dem im Hinblick auf (a) eine oder mehrere Aminosäuren durch andere

25 Aminosäuren ersetzt sind;

(d) ein Polypeptid, in dem im Hinblick auf (a) eine oder mehrere Aminosäuren zur Sequenz hinzugefügt sind; oder

(e) ein Fusions-Polypeptid, das eine Aminosäuresequenz umfaßt, die insgesamt oder teilweise aus der in Figur 2 A und B dargestellten Aminosäuresequenz von menschlichem H1-Prorelaxin als seiner C-terminalen Sequenz und einem Teil eines prokaryontischen Proteins als seiner N-terminalen Sequenz besteht.

21. Isoliertes DNA-Fragment, das ein Polypeptid mit der Aktivität von menschlichem H1-Relaxin codiert, wobei besagtes Polypeptid ausgewählt ist aus der folgenden Gruppe:

(a) ein Polypeptid mit der in Figur 2 A und B dargestellten Aminosäuresequenz, die menschlichem H1-Relaxin entspricht,

35 (b) ein Polypeptid, dem im Hinblick auf (a) eine oder mehrere Aminosäuren fehlen;

(c) ein Polypeptid, dem im Hinblick auf (a) eine oder mehrere Aminosäuren durch andere Aminosäuren ersetzt sind;

(d) ein Polypeptid, in dem im Hinblick auf (a) eine oder mehrere Aminosäuren zur Sequenz hinzugefügt sind; oder

40 (e) ein Fusions-Polypeptid, das eine Aminosäuresequenz umfaßt, die insgesamt oder teilweise aus der in Figur 2 A und B dargestellten Aminosäuresequenz von menschlichem H1-Relaxin als seiner C-terminalen Sequenz und einem Teil eines prokaryontischen Proteins als seiner N-terminalen Sequenz besteht.

45 **Revendications**

1. Fragment d'ADN codant pour la préprorelaxine-H1 humaine, caractérisé en ce qu'il comprend un brin codant et un brin complémentaire correspondant à la séquence complète d'ARNm suivante:

50 AUG CCU CGC CUG UUC UUG CAC CUG CUA GAA UUC UGU UUA  
CUA CUG AAC CAA UUU UCC AGA GCA GUC GCG GCC AAA UGG AAG  
GAC GAU GUU AUU AAA UUA UGC GGC CGC GAA UUA GUU CGC GCG  
55 CAG AUU GCC AUU UGC GGC AUG AGC ACC UGG AGC AAA AGG UCU  
CUG AGC CAG GAA GAU GCU CCU CAG ACA CCA AGA CCA GUG GCA  
GAA AUU GUA CCA UCC UUC AUC AAC AAA GAU ACA GAA ACU AUA  
60 AUU AUC AUG UUG GAA UUC AUU GCU AAU UUG CCA CCG GAG CUG  
AAG GCA GCC CUA UCU GAG AGG CAA CCA UCA UUA CCA GAG CUA  
CAG CAG UAU GUA CCU GCA UUA AAG GAU UCC AAU CUU AGC UUU  
65 GAA GAA UUU AAG AAA CUU AUU CGC AAU AGG CAA AGU GAA GCC

5            GCA GAC AGC AAU CCU UCA GAA UUA AAA UAC UUA GGC UUG GAU  
 ACU CAU UCU CAA AAA AAG AGA CGA CCC UAC GUG GCA CUG UUU  
 GAG AAA UGU UGC CUA AUU GGU UGU ACC AAA AGG UCU CUU GCU  
 AAA UAU UGC UGA

10          2. Fragment d'ADN codant pour la prorelaxine-H1 humaine, caractérisé en ce qu'il comprend un brin codant et un brin complémentaire correspondant à la séquence d'ARNm suivante:

15          10    AAA UGG AAG GAC GAU GUU AUU AAA UUA UGC GGC CGC GAA UUA  
 GUU CGC GCG CAG AUU GCC AUU UGC GGC AUG AGC ACC UGG AGC  
 AAA AGG UCU CUG AGC CAG GAA GAU GCU CCU CAG ACA CCU AGA  
 CCA GUG GCA GAA AUU GUA CCA UCC UUC AUC AAC AAA GAU ACA  
 GAA ACU AUA AUU AUC AUG UUG GAA UUC AUU GCU AAU UUG CCA  
 CCG GAG CUG AAG GCA GCC CUA UCU GAG AGG CAA CCA UCA UUA  
 20          20    CCA GAG CUA CAG CAG UAU GUA CCU GCA UUA AAG GAU UCC AAU  
 CUU AGC UUU GAA GAA UUU AAG AAA CUU AUU CGC AAU AGG CAA  
 AGU GAA GCC GCA GAC AGC AAU CCU UCA GAA UUA AAA UAC UUA  
 25          25    GGC UUG GAU ACU CAU UCU CAA AAA AAG AGA CGA CCC UAC GUG  
 GCA CUG UUU GAG AAA UGU UGC CUA AUU GGU UGU ACC AAA AGG  
 UCU CUU GCU AAA UAU UGC UGA

30          30    3. Fragment d'ADN selon la revendication 2, consistant en un fragment d'ADN bicaténaire codant pour les chaînes peptidiques signal, A, B ou C de la préprorelaxine-H1 humaine, ou pour une combinaison d'au moins deux de ces chaînes, caractérisé en ce qu'il comprend un brin codant et un brin complémentaire correspondant à la séquence d'ARNm appropriée, ou à une combinaison des séquences d'ARNm mentionnées ci-dessous:

35          Peptide signal

40          40    AUG CCU CGC CUG UUC UUG UUC CAC CUG CUA GAA UUC UGU UUA  
 CUA CUG AAC CAA UUU UCC AGA GCA GUC GCG GCC

Chaîne A

45          45    CGA CCC UAC GUG GCA CUG UUU GAG AAA UGU UGC CUA AUU GGU  
 UGU ACC AAA AGG UCU CUU GCU AAA UAU UGC

Chaîne B

50          50    AAA UGG AAG GAC GAU GUU AUU AAA UUA UGC GGC CGC GAA UUA  
 GUU CGC GCG CAG AUU GCC AUU UGC GGC AUG AGC ACC UGG AGC  
 AAA AGG UCU CUG

Chaîne C

55          55    AGC CAG GAA GAU GCU CAG ACA CCU AGA CCA GUG GCA GAA  
 AUU GUA CCA UCC UUC AUC AAC AAA GAU ACA GAA ACU AUA AUU  
 AUC AUG UUG GAA UUC AUU GCU AAU UUG CCA CCG GAG CUG AAG  
 GCA GCC CUA UCU GAG AGG CAA CCA UCA UUA CCA GAG CUA CAG  
 CAG UAU GUA CCU GCA UUA AAG GAU UCC AAU CUU AGC UUU GAA  
 GAA UUU AAG AAA CUU AUU CGC AAU AGG CAA AGU GAA GCC GCA

65

GAC AGC AAU CCU UCA GAA UUA AAA UAC UUA GGC UUG GAU ACU  
CAU UCU CAA AAA AAG AGA

5 4. Procédé de production d'un fragment d'ADN selon l'une quelconque des revendications 1 à 3, caractérisé en ce qu'on cible une banque de clones de gènes en employant comme sonde, un fragment d'ADNc de relaxine porcine ayant la séquence nucléotidique suivante qui correspond aux aminoacides 45 à 95 du peptide C correspondant:

10 GCA GAA ACC ATG CCA TCC TCC ATC ACC AAA GAT GCA GAA  
ATC TTA AAG ATG ATG TTG GAA TTT GTT CCT AAT TTG CCA  
CAG GAG CTG AAG GCA ACA TTG TCT GAG AGG CAA CCA TCA  
15 CTG AGA GAG CTA CAA CAA TCT GCA TCA AAG GAT TCG

5 5. Vecteur de transfert d'ADN, caractérisé en ce qu'il contient des séquences de désoxynucléotides d'ADNc, correspondant à un fragment selon l'une quelconque des revendications 1 à 3.

6. Fragment d'ADN ou vecteur de transfert d'ADN selon l'une quelconque des revendications 1 à 3 et 5, caractérisé en ce qu'un ou plusieurs codons naturels ou leurs ADNc équivalents de ces fragments d'ADN, sont remplacés par un autre codon qui code pour le même aminoacide.

7. Vecteur de transfert d'ADN selon l'une quelconque des revendications 5 et 6, caractérisé en ce qu'il est un plasmide bactérien.

8. Vecteur de transfert d'ADN selon l'une quelconque des revendications 5 à 7, consistant en un ADN de bactériophage.

25 9. Cellule transformée par un vecteur de transfert selon l'une quelconque des revendications 5 à 8.

10. Procédé de préparation d'un vecteur de transfert d'ADN destiné à être employé pour maintenir et répliquer un fragment d'ADN selon l'une quelconque des revendications 1 à 3 et 6, caractérisé en ce qu'on fait réagir la séquence de désoxynucléotides appropriée dudit fragment d'ADN, avec une molécule d'ADN préparée par clivage d'un vecteur de transfert avec une enzyme de restriction.

30 11. Procédé de préparation d'une protéine fusionnée, comprenant une séquence d'aminoacides consistant en totalité ou en partie en la séquence d'aminoacides de la préprorelaxine-H1 humaine formant sa séquence C-terminale, et une partie d'une protéine procaryote formant sa séquence N-terminale, caractérisé en ce qu'on incube un micro-organisme transformé par un vecteur de transfert d'expression comprenant la séquence de désoxynucléotides appropriée.

35 12. Procédé de synthèse de la prorelaxine-H1 humaine comprenant les peptides A et B séparés l'un de l'autre par un peptide C, caractérisé en ce qu'on incube un micro-organisme transformé par un vecteur de transfert et d'expression comprenant un fragment d'ADN codant pour la prorelaxine humaine défini dans la revendication 2, dans des conditions appropriées pour permettre l'expression dudit fragment d'ADN, et on purifie la prorelaxine humaine à partir du lysat ou du milieu de culture de ce micro-organisme.

40 13. Polypeptide ayant l'activité de la préprorelaxine-H1 humaine, ce polypeptide étant choisi parmi le groupe suivant:

(a) un polypeptide ayant la séquence d'aminoacides illustrée sur les figures 2A et 2B, correspondant à la préprorelaxine-H1 humaine;

(b) un polypeptide correspondant au polypeptide (a) avec un ou plusieurs aminoacides en moins;

45 (c) un polypeptide correspondant au polypeptide (a) dans lequel un ou plusieurs aminoacides sont remplacés par des aminoacides différents;

(d) un polypeptide correspondant au polypeptide (a) à la séquence duquel un ou plusieurs aminoacides sont ajoutés; ou

50 (e) un polypeptide fusionné comprenant une séquence d'aminoacides consistant en totalité ou en partie en la séquence d'aminoacides de la préprorelaxine-H1 humaine illustrée sur les figures 2A et 2B formant sa séquence C-terminale, et une partie d'une protéine procaryote formant sa séquence N-terminale.

14. Polypeptide ayant l'activité de la prorelaxine-H1 humaine, ce polypeptide étant choisi parmi le groupe suivant:

55 (a) un polypeptide ayant la séquence d'aminoacides illustrée sur les figures 2A et 2B, correspondant à la prorelaxine-H1 humaine;

(b) un polypeptide correspondant au polypeptide (a) avec un ou plusieurs aminoacides en moins;

(c) un polypeptide correspondant au polypeptide (a) dans lequel un ou plusieurs aminoacides sont remplacés par des aminoacides différents;

60 (d) un polypeptide correspondant au polypeptide (a) à la séquence duquel un ou plusieurs aminoacides sont ajoutés; ou

(e) un polypeptide fusionné comprenant une séquence d'aminoacides consistant en totalité ou en partie, en la séquence d'aminoacides de la prorelaxine-H1 humaine illustrée sur les figures 2A et 2B, formant sa séquence C-terminale, et une partie d'une protéine procaryote formant sa séquence N-terminale.

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15. Polypeptide ayant l'activité de la relaxine-H1 humaine, ce polypeptide étant choisi parmi le groupe suivant:

(a) un polypeptide ayant la séquence d'aminoacides illustrée sur les figures 2A et 2B, correspondant à la relaxine-H1 humaine avec un ou plusieurs aminoacides en moins;

5 (b) un polypeptide ayant la séquence d'aminoacides illustrée sur les figures 2A et 2B correspondant à la relaxine-H1 humaine dans laquelle un ou plusieurs aminoacides sont remplacés par des aminoacides différents;

(c) un polypeptide ayant la séquence d'aminoacides illustrée sur les figures 2A et 2B, correspondant à la relaxine-H1 humaine à la séquence de laquelle un ou plusieurs aminoacides sont ajoutés; ou

10 (d) un polypeptide fusionné comprenant une séquence d'aminoacides consistant en totalité ou en partie, en la séquence d'aminoacides de la relaxine-H1 humaine illustrée sur les figures 2A et 2B, formant sa séquence C-terminale, et une partie d'une protéine procaryote formant sa séquence C-terminale.

15 16. Procédé d'isolement d'un gène chromosomique de relaxine, codant pour une relaxine-H1 humaine selon la revendication 15, caractérisé en ce qu'on cible une banque de gènes en employant comme sonde, une sous-unité de l'ADNc de la relaxine porcine.

17. Séquence d'ADN isolée, comprenant une séquence d'ADN codant pour la préprorelaxine-H1 humaine, la prorelaxine-H1 humaine ou la relaxine-H1 humaine, qui a été modifiée par déletion d'un ou plusieurs des codons naturels, afin de coder pour un peptide comprenant une chaîne A choisie parmi les séquences d'aminoacides de chaîne A 1—24 à 3—24, et une chaîne B choisie parmi les séquences d'aminoacides de chaîne B 1—32 à 3—23, ce peptide ayant l'activité de la relaxine-H1 humaine.

20 18. Séquence d'ADN isolée, comprenant une séquence d'ADN codant pour la prépro:elaxine-H1 humaine, la prorelaxine-H1 humaine ou la relaxine-H1 humaine, qui a été modifiée en remplaçant un ou plusieurs des codons naturels, par des codons qui codent pour des aminoacides autres que ceux codés par le codon naturel, afin de coder pour un peptide comprenant une chaîne A choisie parmi les séquences d'aminoacides de chaîne A 1—24 à 3—24, et une chaîne B choisie parmi les séquences d'aminoacides de chaîne B 1—32 à 4—23, le résidu Met 24 de la chaîne B étant remplacé par un résidu Ala, Val, Gly ou Ser, et ce peptide ayant l'activité de la relaxine-H1 humaine.

25 19. Fragment d'ADN codant pour un polypeptide ayant l'activité de la préprorelaxine-H1 humaine, ce polypeptide étant choisi parmi le groupe suivant:

30 (a) un polypeptide ayant la séquence d'aminoacides illustrée sur les figures 2A et 2B, correspondant à la préprorelaxine-H1 humaine;

(b) un polypeptide correspondant au polypeptide (a) avec un ou plusieurs aminoacides en moins;

35 (c) un polypeptide correspondant au polypeptide (a) dans lequel un ou plusieurs aminoacides sont remplacés par des aminoacides différents;

(d) un polypeptide correspondant au polypeptide (a) à la séquence duquel un ou plusieurs aminoacides sont ajoutés; ou

40 (e) un polypeptide fusionné comprenant une séquence d'aminoacides consistant en totalité ou en partie, en la séquence d'aminoacides de la préprorelaxine-H1 humaine illustrée sur les figures 2A et 2B, formant sa séquence C-terminale, et une partie d'une protéine procaryote formant sa séquence C-terminale.

20. Fragment d'ADN codant pour un polypeptide ayant l'activité de la prorelaxine-H1 humaine, ce polypeptide étant choisi parmi le groupe suivant:

(a) un polypeptide ayant la séquence d'aminoacides illustrée sur les figures 2A et 2B, correspondant à la prorelaxine-H1 humaine;

45 (b) un polypeptide correspondant au polypeptide (a) avec un ou plusieurs aminoacides en moins;

(c) un polypeptide correspondant au polypeptide (a) dans lequel un ou plusieurs aminoacides sont remplacés par des aminoacides différents;

(d) un polypeptide correspondant au polypeptide (a) à la séquence duquel un ou plusieurs aminoacides sont ajoutés; ou

50 (e) un polypeptide fusionné comprenant une séquence d'aminoacides consistant en totalité ou en partie, en la séquence d'aminoacides de la prorelaxine-H1 humaine illustrée sur les figures 2A et 2B, formant sa séquence C-terminale, et une partie d'une protéine procaryote formant sa séquence N-terminale.

21. Fragment d'ADN isolé, codant pour un polypeptide ayant l'activité de la relaxine-H1 humaine, ce polypeptide étant choisi parmi le groupe suivant:

55 (a) un polypeptide ayant la séquence d'aminoacides illustrée sur les figures 2A et 2B, correspondant à la relaxine-H1 humaine;

(b) un polypeptide correspondant au polypeptide (a) avec un ou plusieurs aminoacides en moins;

60 (c) un polypeptide correspondant au polypeptide (a) dans lequel un ou plusieurs aminoacides sont remplacés par des aminoacides différents;

(d) un polypeptide correspondant au polypeptide (a) à la séquence duquel un ou plusieurs aminoacides sont ajoutés; ou

65 (e) un polypeptide fusionné comprenant une séquence d'aminoacides consistant en totalité ou en partie, en la séquence d'aminoacides de la relaxine-H1 humaine illustrée sur les figures 2A et 2B, formant sa séquence C-terminale, et une partie d'une protéine procaryote formant sa séquence N-terminale.

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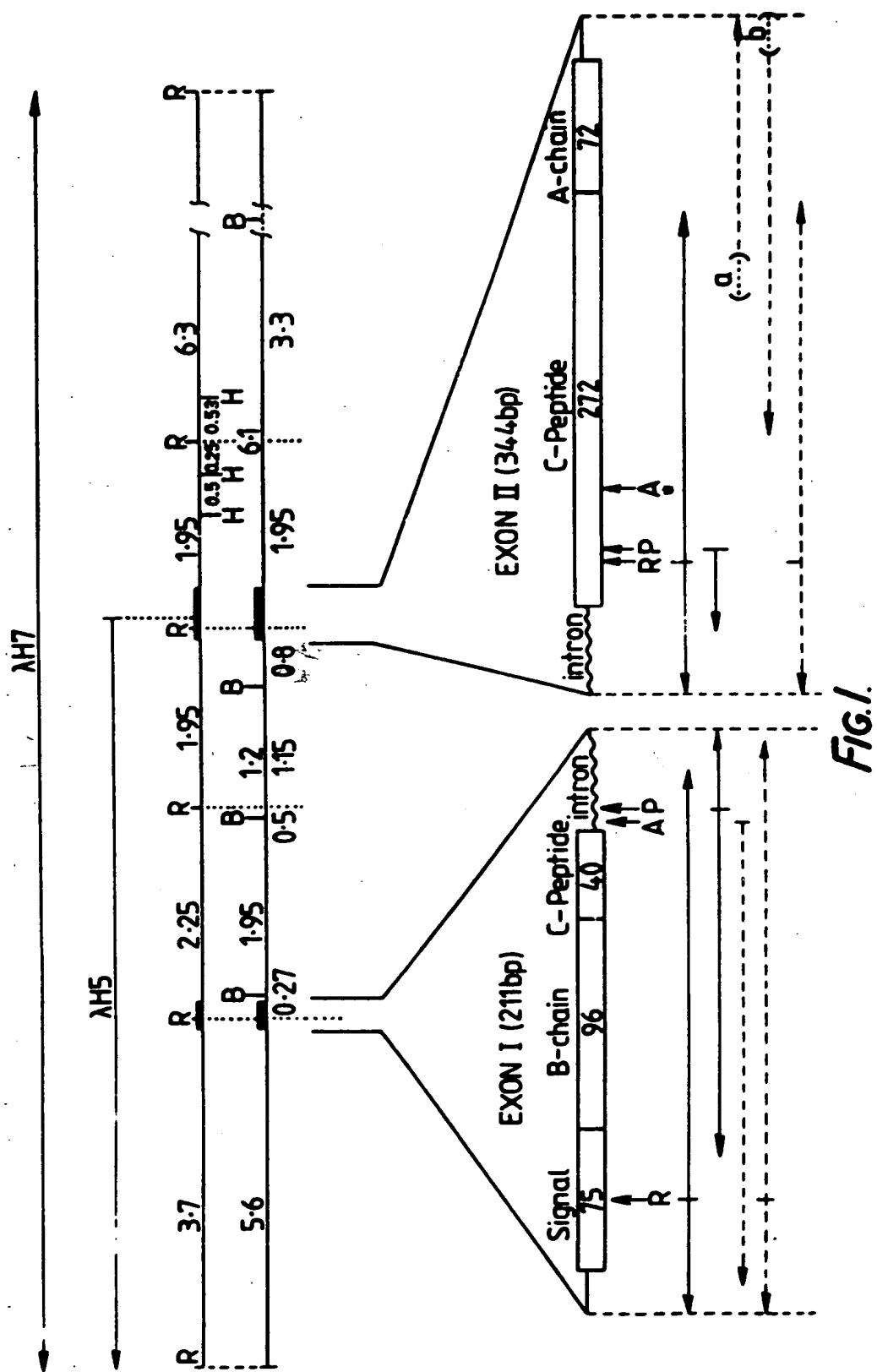
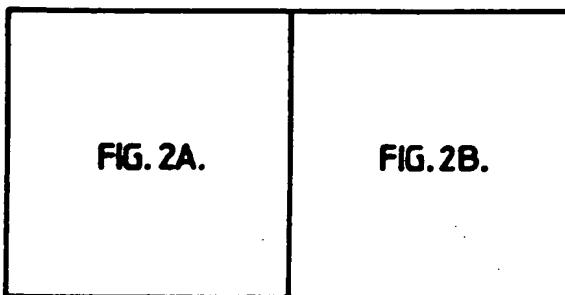
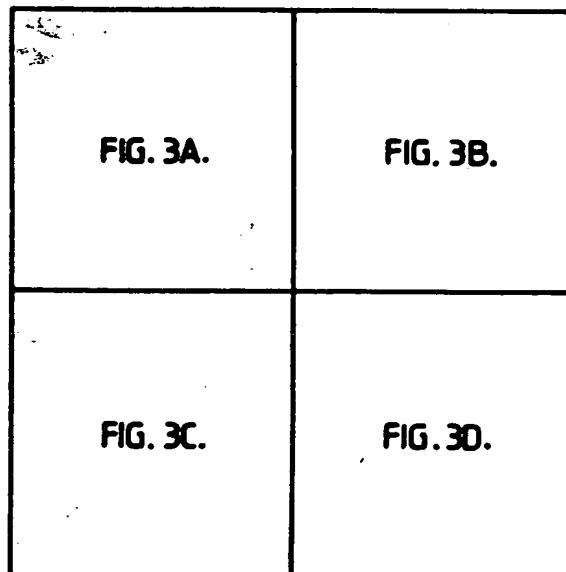


Fig. 1.

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*FIG. 2.*



*FIG. 3.*

—Signal Peptide—

Met Pro Arg Leu Phe Leu Phe His Leu Leu Glu Phe Cys Leu Leu  
AUG CCU CGC CUG UUC UUG UUC CAC CUG CUA GAA UUC UGU UUA CUA 20  
  
Val Ile Lys Leu Cys Gly Arg Glu Leu Val Arg Ala Gln Ile Ala  
GUU AUU AAA UUA UGC GGC CGC GAA UUA GUU CGC GCG CAG AUU GCC 20  
  
Asp Ala Pro Gln Thr Pro Arg Pro Val Ala Glu Ile Val Pro Ser  
GAU GCU CCU CAG ACA AGA CCA GUG GCA GAA AUU GUA CCA UCC 30  
  
Ile Ala Asn Leu Pro Pro Glu Leu Lys Ala Ala Leu Ser Glu Arg  
AUU GCU AAU UUG CCA CCG GAG CUG AAG GCA GCC CUA UCU GAG AGG 80  
  
Asp Ser Asn Leu Ser Phe Glu Glu Phe Lys Lys Leu Ile Arg Asn  
GAU UCC AAU CUU AGC UUU GAA GAA UUU AAG AAA CUU AUU CGC AAU 110  
  
Leu Gly Leu Asp Thr His Ser Gln Lys Lys Arg Arg Pro Tyr Val  
UUA GGC UUG GAU ACU CAU UCU CAA AAA AAG AGA CGA CCC UAC GUG 140  
130  
  
Leu Ala Lys Tyr Cys \*\*\*  
CUU GCU AAA UAU UGC UGA 160

FIG. 2A.

1 | B-chain —  
-10 | 1 |  
Leu Asn Gln Phe Ser Arg Ala Val Ala Ala Lys Trp Lys Asp Asp  
CUG AAC CAA UUU UCC AGA GCA GUC GCG GCC AAA UGG AAG GAC GAU  
Ile Cys Gly Met Ser Thr Trp Ser Lys Arg Ser Leu Ser Gln Glu  
AUU UGC GGC AUG AGC ACC UGG AGC AAA AGG UCU CUG AGC CAG GAA  
Phe Ile Asn Lys Asp Thr Glu Thr Ile Ile Met Leu Glu Phe  
UUC AUU AAC AAA GAA ACA GAA ACU AAU AUU AUU AUG UUG GAA UUC  
Gln Pro Ser Leu Pro Glu Leu Gln Gln Tyr Val Pro Ala Leu Lys  
CAA CCA UCA UUA CCA GAG CUA CAG CAG UAU GUA CCU GCA UUA AAG  
Arg Gln Ser Glu Ala Ala Asp Ser Asn Pro Ser Glu Leu Lys Tyr  
AGG CAA AGU GAA GCC GCA GAC AGC AAU CCU UCA GAA UUA AAA UAC  
Ala Leu Phe Glu Lys Cys Cys Leu Ile Gly Cys Thr Lys Arg Ser  
GCA CUG UUU GAG AAA UGU UGC CUA AUU GGU UGU ACC AAA AGG UCU

FIG. 2B.

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Signal Peptide									
-20									
Met	Pro	Arg	Leu	Phe	Leu	Phe	His	Leu	Glu
AUG	CCU	CGC	CUG	UUC	UUG	UUC	CAC	CUG	Phe
***	**	***	***	***	*	*	**	***	Cys
AUG	CCG	CGC	CUG	UUC	UCC	UAC	CUC	CUA	Leu
Met	Pro	Arg	Leu	Phe	Ser	Tyr	Leu	Leu	Gly
-20									
Lys	Asp	Asp	Val	Ile	Lys	Leu	Cys	Gly	Arg
AAG	GAC	GAU	GUU	AUU	AAA	UUA	UGC	GCG	Glu
**	**	***	**	***	**	*	***	***	Leu
ACG	AAC	GAU	UUU	AUU	AAG	GCA	UGC	GCG	Val
Thr	Asn	Asp	Phe	Ile	Lys	Ala	Cys	Gly	Arg
10									
Lys	Asp	Asp	Val	Ile	Lys	Leu	Cys	Gly	Arg
AAG	GAC	GAU	GUU	AUU	AAA	UUA	UGC	GCG	Glu
**	**	***	**	***	**	*	***	***	Leu
ACG	AAC	GAU	UUU	AUU	AAG	GCA	UGC	GCG	Val
Thr	Asn	Asp	Phe	Ile	Lys	Ala	Cys	Gly	Arg
30									
Arg	Ser	Leu	Ser	Gln	Glu	Asp	Ala	Pro	Gln
AGG	UCU	UGC	AGC	CAG	GAA	GAU	GCU	CCU	Thr
**	**	**	***	**	***	**	**	**	Pro
ACU	GCU	CUC	AGC	CUG	GAA	GAG	CCU	CAG	Ala
Thr	Ala	Leu	Ser	Leu	Glu	Glu	Pro	Gln	Gln
40									
Asp	Ala	Pro	Gln	Thr	Pro	Arg	Pro	Ala	Arg
GAU	GCU	CCU	CAG	ACA	CCU	AGA	CCA	CCU	CCA
**	*	*	*	*	*	**	**	**	**
GAG	CCU	CAG	CUG	GAA	ACU	GGA	ACU	GGA	CCC
Glu	Pro	Gln	Leu	Thr	Thr	Gly	Gly	Gly	Pro
Intron (3.7kb)									
----- cacatcacatTTTTTTTta									

FIG. 3A.

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-10												B-chain				
Leu	Leu	Asn	Gln	Phe	Ser	Arg	Ala	Val	Ala	-1	Ala	1	Lys	Trp		
CUA	CUG	AAC	CAA	UUU	UCC	AGA	GCA	GUC	GIG		GCC	AAA	UGG			
**	***	*	***	**	**	***	*	*	*		**	*	*	*	*	*
CUC	CUG	AGC	CAA	CUU	CCC	AGA	GAA	AUC	CCA		GGC	CAG	AGU			
Leu	Leu	Ser	Gln	Leu	Pro	Arg	Glu	Ile	Pro		Gly	Gln	Ser			
										-1		1				

Ala	Gln	Ile	Ala	Le	Cys	Gly	Met	Ser	Thr	Trp	Ser
GCG	CAG	AUU	GCC	AUU	UGC	GGC	AUG	AGC	ACC	UGG	AGC
*	*	*	*	**	**	***	*	**	**	***	AAA
CUG	UGG	GUG	GAG	AUC	UGU	GCG	UCC	CUC	UCC	UGG	GGA
Lys	Trp	Val	Glu	Ile	Cys	Gly	Ser	Val	Ser	Trp	Arg

U	Ile	Val	Pro	Ser	Phe	Ile	Asn	Lys	Asp	Thr
AA	Alu	GUU	CCA	UCC	UUC	AUC	AAC	AAA	GAU	ACA
**	*	*	***	***	*	***	*	***	***	**
AA	ACC	AUG	CCA	UCC	UCC	AUC	ACC	AAA	GAU	GCA
U	Thr	Met	Pro	Ser	Ser	Ile	Th	Lys	Asp	Ala
				50						

FIG. 3B.

60								70							
Glu	Thr	Ile	Ile	Ile	Met	Leu	Glu	Phe	Ile	Ala	Asn	Leu	Pro		
GAA	ACU	AUA	AAU	AUC	AUG	UUG	GAA	UUC	AUU	GCU	AAU	UUG	CCA		
***	*	**	*	*	***	***	***	**	**	**	***	***	***		
GAA	AUC	UUA	AAG	AUG	AUG	UUG	GAA	UUU	GUU	CCU	AAU	UUG	CCA		
Glu	Ile	Leu	Lys	Met	Met	Leu	Glu	Phe	Val	Pro	Asn	Leu	Pro		
90															
Leu	Pro	Glu	Leu	Gln	Gln	Tyr	Val	Pro	Ala	Leu	Lys	Asp	Ser		
UUA	CCA	GAG	CUA	CAG	CAG	UAU	GUU	CCU	GCA	UUA	AAG	GAU	UCC		
*	*	***	***	**	**			**	***	**	***	***	***		
CUG	AGA	GAG	CUA	CAA	CAA			UCU	GCA	UCA	AAG	GAU	UCG		
Leu	Arg	Glu	Leu	Gln	Gln			Ser	Ala	Ser	Lys	Asp	Ser		
120															
Arg	Gln	Ser	Glu	Ala	Ala	Asp	Ser	Asn	Pro	Ser	Glu	Leu	Lys		
AGG	CAA	AGU	GAA	GCC	GCA	GAC	AGC	AAU	CCU	UCA	GAA	UUA	AAA		
***	***	*	***	**	**	***	*	**	*	**	***	***	***		
AGA	CAA	AAU	GAA	GCA	GAA	GAC	AAA	AGU	CUU	UUA	GAA	UUA	AAA		
Arg	Gln	Asn	Glu	Ala	Glu	Asp	Lys	Ser	Leu	Leu	Glu	Leu	Lys		
140								150							
Pro	Tyr	Val	Ala	Leu	Phe	Glu	Lys	Cys	Cys	Leu	Ile	Gly	Cys		
CCC	UAC	GUG	GCA	CUG	UUU	GAG	AAA	UGU	UGC	CUA	AUU	GGU	UGU		
*	**	**	***			***	***	***	**	**	*	***	***		
UUC	CGU	AUG	ACA	CUG	AGC	GAG	AAA	UGU	UGC	CAA	GUU	GGU	UGU		
Phe	Arg	Met	Thr	Leu	Ser	Glu	Lys	Cys	Gys	Gln	Val	Gly	Cys		
A-chain															

FIG. 3C.

80											
Pro	Glu	Leu	Lys	Ala	Ala	Leu	Ser	Glu	Arg	Gln	Pro
CCG	GAG	CUG	AAG	GCA	GCC	CUA	UCU	GAG	AGG	CAA	CCA
**	***	***	***	***	*	*	***	***	***	***	***
CAG	GAG	CUG	AAG	GCA	ACA	UUG	UCU	GAG	AGG	CAA	CCA
Gln	Glu	Leu	Lys	Ala	Thr	Leu	Ser	Glu	Arg	Gln	Pro
100											
Asn	Leu	Ser	Phe	Glu	Glu	Phe	Lys	Lys	Ile	Arg	Asn
AAU	CUU	AGC	UUU	GAA	GAA	UUU	AAG	AAA	AUU	CGC	AAU
***	***	*	***	***	***	***	***	***	***	*	**
AAU	CUU	AAC	UUU	GAA	GAA	UUU	AAG	AAA	AUU	CUU	AAC
Asn	Leu	Asn	Phe	Glu	Glu	Phe	Lys	Lys	Ile	Ile	Leu
100											
Tyr	Leu	Gly	Leu	Asp	130	Thr	His	Ser	Gln	Lys	Lys
UAC	UUA	GGC	UUG	GAU	ACU	ACU	CAU	UCU	CAA	AAA	AAA
**	***	**	**	***	*	***	***	**	*	***	***
AAC	UUA	GGU	UUA	GAU	AAA	CAU	UCC	AGA	AGA	AAA	AAA
Asn	Leu	Gly	Leu	Asp	Lys	His	Ser	Arg	Arg	AGA	AGA
130											
Thr	Lys	Arg	Ser	Leu	Ala	Lys	Tyr	Cys	Arg	Arg	Arg
ACC	AAA	AGG	UCU	CUU	GCU	AAA	UAU	UGC	CGA	CGA	CGA
**	**	*	*	**	***	**	***	***	***	***	***
AUC	AGA	AAA	GAU	AUU	GCU	AGA	UUA	UGC	CUG	CUG	CUG
Ile	Arg	Lys	Asp	Ile	Ala	Arg	Leu	Cys	Leu	Lys	Arg
150											
160											

FIG. 3D.

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